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COMPARISON OF HUMAN AND ANIMAL ROTAVIRUS ISOLATES

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LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

PART I: GENERAL INTRODUCTION

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This introduction will cover some of the historical aspects of human and animal infantile gastroenteritis outlining the discovery of rotavirus and will discuss the relationship of human and animal rotavirus isolates. It will not cover the developments and investigations during the actual time period of this study (1978-1981). These developments will be dealt with in the introduction and discussion sections of the three main chapters and in the general discussion.

1.1 Diarrhoeal Diseases

In developing countries diarrhoeal diseases compete with acute respiratory infections as the leading cause of childhood mortality. It is believed that every year up to 1,000 million diarrhoeal episodes cause 4-5 million deaths in young children (WHO Chronicle, 1978). Diarrhoeal diseases are the single major killer of under five-year olds, being responsible for one-third or more of all deaths in this age group. In 1975, approximately 500 million episodes of diarrhoeal cases in children under five years of age were reported in Asia, Africa and Latin America alone, causing from 15 to 18 million deaths. It has been estimated that more than one-third of the beds in childrens hospitals and wards in developing countries are occupied by diarrhoeal cases. In a community study, children were found to spend 2 months per year (16%) with diarrhoea and in another study it was found that children spending more than 5% of days in a year with diarrhoea were found to falter in weight and height gain. There has been a close association shown between diarrhoea and malnutrition. Malnounrished children have a higher incidence of severe diarrhoea resulting in a higher rate of diarrhoea related mortality. Meanwhile diarrhoeal diseases are probably the most important of all childhood diseases as a cause of malnutrition

because of food withdrawal by mothers and through malabsorption. In non-epidemic seasons it has been shown that cholera accounts for less that 5 to 10% of all acute diarrhoea in cholera endemic areas (WHO Chronicle, 1978). Diarrhoeal diseases of the young are therefore a serious problem in all countries and especially developing countries. There has been much research into the clinical and aetiological aspects of diarrhoeal diseases and they will continue to be a major field of interest in world health.

The aetiology of infantile diarrhoea has received considerable study over the last few years. A number of bacterial and viral agents have been isolated that are known to cause acute diarrhoea of children (infantile gastroenteritis). More traditional enteric bacterial pathogens include salmonellae, shigellae, vibrios and enteropathogenic E. coli. These are known to cause outbreaks of infantile gastroenteritis but their overall incidence in acute infantile diarrhoea is not known, however, it is suspected to be relatively low. It has been estimated that in temperate climates salmonellae, shigallae and enteropathogenic E. coli together account for less than 10% of all infantile gastroenteritis cases. This incidence is likely to be higher in developing tropical countries where lack of public hygiene facilities allows for a greater build up of these pathogens. Additional bacterial pathogens include toxin producing Staphylococcus aureus, Vibrio parahae.molyticus, Bacillus cereus and Clostridium perfringens. More recently enterotoxigenic E. coli have been shown to be of importance as a cause of infantile diarrhoea causing symptoms similar to cholera. It is alse found in adult diarrhoea and has been implicated in cases of travellers' diarrhoea. Yersinia enterocolitica and Campylobacter jejuni have been only recently recognized as pathogens causing acute diarrhoea. They have unusual media and isolation requirements and have not yet been adequately investigated as to their role in diarrhoeal diseases in many countries (WHO Chronicle, 1978).

Gastroenteritis in childhood as can be seen by the information already given is a serious problem and one of enourmous importance, particularly in developing countries. Despite its significance, up until the early 1970s, known bacterial pathogens could only be identified in the stools of less than 20% of children with acute diarrhoea in developed countries and less than 50% in most developing countries (Walker-Smith, 1978).

"Viral gastroenteritis" was a term that was often used to describe cases of acute gastroenteritis in children from which a specific bacterial pathogen could not be cultured. Studies of faecal viruses usually showed similar levels of the same viruses in gastroenteritis patients and normal children. Adeno, coxsackie, polio and ECHO (11, 14 and 18) viruses have all been associated with occasional outbreaks of infantile gastroenteritis but their isolation rate in disease cases is low (Bishop, et al., 1976). In 1978 an outbreak of gastroenteritis occurred in a school in Norwalk, USA. Over the period of the outbreak 50% of the children and teachers experienced a bout of the illness. An attempt was made to isolate a virus from a faecal specimen. After three passages in organ culture, fluid was given to four human volunteers who after 48 hours showed symptoms similar to those of patients in the original outbreak. The virus that was identified in these experiments has been named Norwalk virus and is thought to be a member of the parvovirus group (Hodes, 1980). A number of other small viruses have been isolated from gastroenteritis outbreaks that are morphologically similar to Norwalk virus but antigenically distinct. In 1970 there were gastroenteritis outbreaks in Montgomery County and Hawaii, USA. Clinically and epidemiologically these two outbreaks resembled Norwalk virus outbreaks, small virus particles (26-27 mm) were found in the stools of patients. The Norwalk and Hawaii viruses, however, are antigenically quite distinct. The Norwalk and Montgomery County viruses do share some antigens but are not identical. Two outbreaks of gastroenteritis in

Australia in 1977 and 1978 were caused by a small virus, one outbreak was in a primary school and the other was nationwide as a result of oyster contamination with sewage. The oyster-borne agent appeared to be the same as Norwalk agent morphologically and antigenically whereas the primary school outbreak was caused by a virus of smaller size (22-25 nm). Outbreaks with small viruses antigenically distinct from Norwalk virus and the Hawaii virus have also occurred in England (Hodes, 1980).

A number of other viruses have been implicated in outbreaks of gastroenteritis. It is probable that they play a role in the aetiology of human gastroenteritis but their relative importance is unclear. These viruses include adeno-, astro-, calici- and corona-like viruses. It is known that astroviruses and caliciviruses regularly cause acute human gastroenteritis (Hodes, 1980).

These bacterial and viral agents however, do not account for the majority of infantile gastroenteritis cases that occur. They are important and can be the major cause of gastroenteritis in particular outbreaks but do not explain the vast numbers of cases that occur every year and that follow a winter seasonal pattern in temperate countries throughout the world.

In 1973, Bishop, et al., in Melbourne, Australia and Flewett, et al., in Birmingham, England, within the space of three weeks of each other found virus particles in duodenal mucosa biopsy and human stool specimens respectively. This virus, now known as rotavirus, has come to be recognized as the major cause of infantile gastroenteritis throughout the world, accounting for approximately 50% of all cases and up to 80% at peak periods of seasonal epidemics.

1.2 Historical Perspective

Although Bishop, et al., and Flewett, et al., will be recorded as

being the discoverers of rotavirus, its history goes right back to the 1930's and perhaps even further. The concept that infantile gastroenteritis might be caused by a viral agent began in the 1930's when outbreaks of diarrhoea occurred in hospital nurseries amongst newborn infants throughout the United States and in Europe. In very few of these outbreaks could a particular causative bacterial agent be isolated and so the concept that a virus was responsible naturally arose.

In 1941, Light and Hode began studies to isolate a virus that caused gastroenteritis. In an outbreak that occurred in the premature infant nursery in Baltimore, USA, in 1942, specimens were inoculated into a three-week old calf. After an incubation period of 2 days the calf became ill with symptoms of severe diarrhoea, with mucous and blood in the stools. Twenty-eight further passages were made in calves by inoculation of faecal material and in every case a diarrhoeal illness occurred, and in 15% of the cases, calves failed to survive the disease. In a similar outbreak four months later, equivalent experiments were conducted with similar results. However, to say that the virus isolated was in fact the causative agent of infantile gastroenteritis, a temporal immunologic and epidemiologic relationship of the virus to the illness had to be shown. This was subsequently achieved. Virus was shown to be absent from healthy infants but readily isolated from patients with diarrhoea. Neutralizing antibodies were not found in patients at the onset of illness but appeared during convalescence. The virus caused disease in inoculated calves and was excreted in the stools of infected animals, whereas uninoculated calves did not excrete the virus. Neutralizing antibodies were absent in calves prior to inoculation but occurred during convalescence. Calves that had recovered from naturally acquired diarrhoea were still susceptible to the human virus indicating that the agent was different from that which caused scours in local herds. Thus, Light and Hodes were the first to isolate a virus from human gastroenteritis patients and establish that it was in fact the causative

agent of the disease. In 1974, Hode took a frozen lyophillized specimen of calf stool from a calf that had developed diarrhoea after inoculation with an infant stool filtrate in 1943, to Drs. Wyatt and Kapikian and showed by electron microscope that the 32 year old specimen contained particles the same size as rotavirus and had the characteristic wheel-like morphology. Dr. Wyatt also did antigenic studies on the virus by immunoassays and showed that it was in fact rotavirus. Not surprisingly, it was not capable of inducing disease when inoculated into a calf (Hodes, 1980).

After Hodes initial studies in 1942 very little research on viral infantile gastroenteritis occurred for a further 25 years. It was left to the animal virologists to pioneer further developments in the establishment of rotavirus as a causative agent of gastroenteritis of the young.

Outbreaks of acute diarrhoea in suckling mice were reported from several parts of the USA in the early 1940's but a causative agent was not isolated. The disease appeared to be seasonal occurring mainly in the autumn and early winter months and appeared within the first 2 weeks of life. Mice usually recovered within a few days.

In a series of studies from 1957 to 1966, Elizabeth Kraft established that the infant mice disease was caused by a heat-resistant virus which was highly infectious, with an incubation period of 40 hours to 10 days. The virus was ether-resistant and was 65-75 nm in diameter as measured by its filtration end point. It was further characterized by Much and Zajac in 1972 as containing RNA and having a capsid that resembled bluetongue virus without its outer layer. This virus came to be known as the epizootic diarrhoea of infant mice virus (EDIM).

In 1958 the SA_{11} virus was isolated from a rectal swab of a vervet monkey. A similar virus that has come to be known as the "O" virus, was isolated from South Africa on five occasions from pooled filtrate washings of cattle and sheep intestines at the Johannesburg municipal abbatoir. Its

species of origin is unknown as pigs and horses were also slaughtered in this abattoir (Hodes, 1980).

Diarrhoea of newborn calves has for centuries been a serious problem among stock breeders. It was not until studies were done using gnotobiotic animals that success came in isolating a causative viral agent. Mebus, et al., in 1969 first succeeded in transmitting infection by inoculating calves with filtrates of diarrhoeal faeces. Virus particles, 65 nm in diameter, were found in large numbers in the faeces of the infected animals. The work was confirmed in England where two strains were adapted to grow in tissue culture (Flewett and Woode, 1978).

It took some time for human virologists to catch up with their counterparts in the veterinary world. Not until 1973 when Bishop, et al., and Flewett, et al., reported their discovery of virus particles in human specimens. Since then this virus, subsequently called rotavirus, has been identified in almost every country of the world and is known to be the major contributor of infantile gastroenteritis in developed countries and is a major cause of infantile diarrhoea in developing countries also.

Subsequent to the discovery of rotaviral particles in diarrhoeal faeces, a large amount of research has gone into the characterization of rotavirus.

1.3 Characterization of Rotavirus

1.3.1 Rotavirus Morphology. When viewed by electron microscopy, rotavirus particles have a distinctive morphology. They appear as either double- or single-shelled, spherical particles. The double-shelled particles have a very definite edge whereas single-shelled particles could be described as having a rough, diffuse border. There have been varying estimates of the size of the virus particle ranging from 65 to 80 nm in diameter (Martin, et al., 1975). The virus particle is made up of an icosahedral

core to which are attached, in a radial arrangement, capsomeric subunits which form the inner capsid layer. Surrounding this is an outer layer which has a smooth border on the end of which appear to be short "spikes". There is some disagreement as to the capsomeric arrangement of the two capsid layers. Martin, et al. (1975) reported that the virus surface was composed of 32 large capsomeres arranged in a T=3 pattern, with a structure similar to that of the orbiviruses. Each capsomere was composed of 180, trimeric subunits arranged as for a T=9 icosadeltahedron. However, Esparza and Gil (1978) proposed an alternative model based on their E.M. studies. They suggested that the inner capsid was composed of morphological subunits arranged as an open mesh around 162, 5 and 6-coordinated holes, formed by the icosahedral arrangement of 320 subunits. Although they could not directly show the structure of the outer capsid they suggested that it followed the same symmetry as the inner shell. A similar model was proposed by Stannard and Schoub (1977) for the structure of EDIM and SA_{11} rotaviruses although their model postulated that there were only 92 holes in a capsid layer. The differences in the proposed models are probably due to the different methodology employed. Different support membranes and stains used for E.M. examination give different resolution of virus particles and their substructure.

It is well agreed that the density of rotavirus particles is 1.36 to 1.38 with the complete, double-shelled particles having a density by CsCl gradient centrifugation of 1.36 gm/ml and the incomplete, single-shelled particles 1.38 gm/ml (Flewett and Woode, 1978).

Further characterization of rotaviruses has been undertaken at the molecular level by electrophoretic separation of RNA segments and structural polypeptides.

1.3.2 Rotaviral RNA. Welch in 1971 and again in 1973, in an analysis of the genome of calf rotavirus showed that it consisted of

double-stranded (ds) RNA. This was confirmed by Rodger, et al., in 1975 who also showed that the genome could be resolved into eleven segments by polyacrylamide gel electrophoresis (PAGE). Thus rotaviruses appeared to be very similar to reovirus and orbivirus. RNA "fingerprinting", or the separation of RNA segments by PAGE has subsequently been done for a number of different rotaviral isolates and has become one of the favoured techniques for characterizing rotaviral isolates. By 1978, PAGE of the RNA genome had been resolved for rotaviruses from calves, humans, piglets, lambs and the SA_{11} virus (for relevant papers see Rodger, et al., 1975; Kalica, et al., 1976; Todd and McNulty, 1977 and Rodger, et al., 1977). The majority of these reports found the genome to be composed of 11-12 RNA segments, although Obijeski, et al., (1977) found the human rotavirus to be made up of as many as 15 segments. However, the weight of evidence is in favour of there being only 11 segments in the rotaviral double-stranded RNA genome. There has also been some disagreement over the molecular weight estimates of the genome but this probably reflects the different types of techniques used. The total molecular weight appears to be in the order of 12×10^6 daltons with segments ranging from 2.2 to 0.2×10^6 in size (Rodger, et al., 1975).

1.3.3 Rotaviral Polypeptides. The rotavirus particle appears to be made up of 8 to 9 structural polypeptides (Rodger, et al., 1975). For lamb rotavirus it was found that four of the polypeptides were present in complete virions only indicating that they were present in the outer capsid layer (Todd and McNulty, 1977). Rodger, et al., (1977) also found 4 polypeptides in the outer capsid and that the major polypeptide in this layer was glycosylated. When the calf and human rotavirus isolates were compared by electrophoretic migration of their structural polypeptides, a distinct difference was evident (Rodger, et al., 1975). Molecular weights for the nine polypeptides differentiated for calf rotavirus are estimated to range

from 131 to 32 x 10^3 for the inner layer and from 58 to 14.5 x 10^3 for the outer layer (Todd and McNulty, 1977).

1.3.4 Other Characteristics. Rotavirus has been found to be a very stable virus. Palmer, et al., (1977) found human rotavirus particles to be stable to almost all of the physical and chemical treatments they tried. The morphology of virus particles was unchanged after treatment with heat, high speed centrifugation, high salt concentrations, extremes of pH, chymotrypsin, papain, pepsin and nonionic detergents. At a pH lower than 3 virions lost their outer capsid but otherwise remained whole. When treated with trypsin-EDTA (0.125%) particles were completely degraded after 2 hours incubation at 37°C. The relevance of trypsin degradation to infectivity and antigenicity is discussed in the general discussion. Calf rotavirus is also known to be resistant to lipid solvents (Welch and Thompson, 1973).

1.4 The Relationship of Human and Animal Rotaviral Isolates

Rotavirus has been isolated from many different host species. By 1978 it had been found in mice, calves, humans, lambs, piglets, rabbits, deer and pronghorn antelope (Flewett and Woode, 1978). It soon became important to consider whether rotavirus isolates were able to infect hosts of more than one species and in particular whether humans were able to acquire a rotaviral infection from an animal host. This obviously has serious implications in the epidemiology of rotaviral infections and in considerations of possible control measures. For vaccination to be employed as a means of protecting the young from rotaviral infection it needs to be considered whether humans can be infected by more than one type of virus and whether the virus is able to cross host barriers. Such considerations have led to a number of studies of comparisons of human and animal rotavirus at both the genetic and antigenic level.

The morphological and serological observations of Flewett, et al., (1974) suggested that human and calf rotaviruses were related antigenically as well as being of identical morphology. They suggested that the two viruses shared a common antigen on the internal capsid. This was verified and developed further by Woode, et al., (1976) who were able to show that the inner capsid of rotavirus isolated from pigs, foals, mice, monkeys, children, calves and of the "O" agent were all able to react serologically with convalescent sera from children, calves, mice, piglets, and foals. However these same virus isolates, when tested via their outer capsid reacted to different degrees using the same series of convalescent sera. There was also a difference exhibited by the sera to neutralise the growth of a tissue culture-adapted calf rotavirus. The conclusion from these experiments is that all rotaviruses appear to be related antigenically at the inner capsid level but differ in their serological relationship at the outer capsid.

This antigenic relationship of a common group antigen has proved useful in the diagnosis of rotavirus infections. It has meant that reference antisera could be prepared against tissue culture adapted rotavirus strains such as Nebraska calf diarrhoea virus (NCDV), Northern-Ireland calf rotavirus (NI), and SA_{11}; also tissue culture grown rotavirus could be used as antigen when detecting specific antirotavirus antibodies in sera. Kapikian, et al., (1976) showed that NCDV could be used as substitute antigen in complement fixation tests for detecting specific antirotavirus antibodies in cases of human infection. They also showed that SA_{11}, "O" agent and EDIM rotavirus could be used as substitute antigens with perhaps the "O" virus being the most efficient.

Yolken, et al., (1978) utilized the antigenic diversity of the outer capsid of rotavirus to distinguish between isolates of different host origin. By using post-infection antisera they were able to block activity to

different degrees in an enzyme linked immunosorbant assay (ELISA) blocking test. Rotavirus isolates from humans were distinguishable from those of calf, piglet, foal, monkey and infant mice origin. Post-infection sera blocked the ELISA in the homologous situation (e.g., human sera vs human rotavirus) to a greater extent than it did in the heterologous (e.g., calf sera vs human rotavirus). These studies showed that there are a number of types of rotavirus from animal and human hosts that are distinguishable at the antigenic level but that difference is only one of degree of reactivity. Such antigenic diversity does not indicate whether or not a viral isolate would be able to infect a number of host types.

Because of the interest in the host range of rotaviral isolates a number of transmission studies between different host species have been conducted. In 1975, Middleton, et al., were able to infect germfree and conventional piglets with rotavirus from stools of infants with acute gastroenteritis. Virus was found subsequently in stools and mucosal epithelial cells of the small intestine. Some of the piglets developed a mild diarrhoea and a serological conversion. During the study, control piglets were housed in close proximity to the experimentally infected animals. Twice during the study these control piglets also became infected with the only link between the two groups of animals being the animal handlers. This indicates that it is possible for piglets to naturally acquire rotavirus under field conditions from a human source. Torres-Medina, et al., (1976) orally inoculated 1-4 day old gnotobiotic piglets with rotavirus from human infants. Piglets developed diarrhoea 2 to 7 days post inoculation. This virus was able to be serially passaged five times in piglets with a total of 19 out of 21 developing diarrhoea. In the same year Tzipori in Australia (1976) passaged human rotavirus in gnotobiotic piglets and then fed the virus orally to 2-week old pups. Virus excreted from these pups was then passaged in 6-week old pups. Virus particles were

detected in the faeces of the inoculated and of the uninoculated pups that had been housed in close proximity. At no stage was clinical diarrhoea or any illness noted in the pups. There is, therefore, at least one strain of rotavirus, of human origin, which is capable of propagating in pups producing large quantities of virus in faeces without causing any clinical disease. Rotavirus isolated from calves and foals have also been shown to be infectious for piglets (Woode, 1976).

Woode and Crouch (1978) and Woode, et al., (1978) showed that cross protection antibodies were not reliable in studies with calves. In both studies inoculation of gnotobiotic calves with human and foal rotavirus failed to protect against infection when subsequently infected with bovine rotavirus. However primary inoculation with a U.S. strain of bovine rotavirus did give complete protection against subsequent infection with a U.K. strain of bovine rotavirus.

It can be concluded from these transmission and cross protection studies of the different rotaviral isolates that strains from different host types do not give full protection against rotaviral infection with another type and even different rotaviruses from the same host species may not afford complete cross protection (Rodriguez, et al., 1978). Human rotaviruses are naturally able to infect both piglets and pups and possibly other animal types. Together these results may indicate that rotaviruses naturally pass from one host species to another, at times only producing mild or asymptomatic infections due to antigenic similarity to a previous rotavirus exposure giving some cross protection.

The question that was in everyones mind was, "human rotaviruses can infect animal hosts but can animal rotaviruses infect young children in return and cause a symptomatic infection"? Probably many wondered whether animal rotavirus isolates could be the source of a "new" rotavirus type for a human population causing the recurrent winter epidemics so often observed Also with rotaviruses having a segmented genome was it possible

that there may be reassortment of genetic segments between the different types consequently giving a changing population of rotaviral types rather than a static number as it was first thought? Such a situation may mean that rotaviral epidemics each winter are caused by either minor changes in the rotaviral genome due to random mutations reflected in a minor change in antigenicity or by more major changes due to the acquirement of a new genetic segment via genetic reassortment, analogous to the minor "drift" and major "shift" mechanisms operating in influenza viruses.

The major objectives of this study were to determine more precisely the degree of relationship of rotaviral isolates from different host species by comparison at the genetic level. Before this could be fully investigated, suitable methods for the detection of rotavirus in a large number of specimens from a number of different host species had to be developed. Part II will describe the development of these techniques and particularly the enzyme-linked immunosorbant assay (ELISA). Part III will describe the extraction and comparison of genetic dsRNA from rotaviral isolates by polyacrylamide gel electrophoresis and the more precise comparison of this genetic material by molecular hybridization will be described in Part IV.

PART II: DETECTION OF ROTAVIRUS INFECTION IN HUMAN AND ANIMAL HOSTS

PART II: DETECTION OF ROTAVIRUS INFECTION IN HUMAN AND ANIMAL HOSTS

1. INTRODUCTION - DETECTION METHODS

The need for detection and identification of viruses from faeces, causing specific diseases, has led to the development of a number of useful and rapid diagnostic techniques. For years infantile gastroenteritis epidemics were thought to be caused by a virus but traditional cell culture methods only led to the isolation and identification of viruses from faeces which were not regularly implicated in cases of diarrhoea. With the development of electron microscopy of faecal specimens however, specific viruses have been detected in association with diarrhoeal disease. Bishop, et al., (1973) used electron microscopy to detect rotavirus particles in epithelial cells of duodenal mucosa from children with acute gastroenteritis. Since this initial discovery a number of techniques have been developed in an attempt to rapidly identify rotavirus in an acute disease situation. Some of the techniques developed and used in routine diagnosis of rotavirus are: immunofluorescence (Davidson, et al., 1975), complement fixation (Kapikian, et al., 1975), counterimmunoelectrophoresis (Spence, et al., 1975), radioimmunoassay (Watanabe, et al., 1978), enzyme-linked immunosorbant assay (Yolken, et al., 1977) and serum neutralization (Beards, et al., 1980).

There are certain advantages and disadvantages inherent in all these techniques that set limits on their use and makes some techniques more applicable to certain situations. This section of the thesis will cover the limits and application of some of these techniques, their development for survey of rotavirus incidence and the results of that survey.

1.1 Electron Microscopy

The availability of electron microscopes in many virology departments has led to the development of its use as a diagnostic tool. Initially

identification of viruses was by morphological characteristics alone as first used by Peters, et al., (1962) in the investigation of an outbreak of smallpox in West Germany. It has subsequently been used in diagnosing a virus aetiology in a number of human, animal and plant diseases.

Rotaviruses were first discovered in Australia (Bishop, et al., 1973) and England (Flewett, et al., 1973) using electron microscopes. Rotaviruses have a characteristic cart-wheel appearance from which their name has been derived. Electron microscopy gives a specific and rapid answer in acute cases of rotaviral infantile gastroenteritis. The main method has been to make a suspension of faecal material which is clarified by low speed centrifugation and then a drop of the supernatant is dried onto formvar or carbon coated grids and negatively stained with phosphotungstic acid or ammonium molybdate. When dry the grid is then examined by EM.

A further development in the use of the EM was to use immune sera to agglutinate virus particles (Gardner, 1977). However in a comparative study of EM, immune electron microscopy (IEM) and complement fixation (CF) by Zissis, et al., (1978), it was found that IEM did not render the diagnosis any easier nor did it increase the number of specimens found positive by EM. It is known that at least 10^6 virus particles per gram of faeces is necessary before virus can be detected. Because of this insensitivity, screening of a large number of specimens may lead to a high number of false negative results. In a comparative study of EM with immuno-electro-osmophoresis (IEOP) by Grauballe, et al., (1977) it was found that by IEOP, 61% of the samples contained rotavirus antigen whereas by EM only 50% were diagnosed as positive.

Another disadvantage of EM is the time involved in obtaining a diagnosis. For one or two specimens it is very rapid, taking only 15-30 minutes to obtain a result. Because each specimen has to be handled individually, for screening of large numbers of specimens the time factor becomes very

significant. EM's are also very expensive and consequently many areas have no access to such equipment. It was because of these main disadvantages: insensitivity, cost and unsuitability for screening large numbers of specimens, that other rapid techniques were developed.

Initially until other tests were developed we routinely used EM to diagnose rotavirus specimens and even after development of other assay systems, EM was used as a confirmationary test for many specimens.

1.2 Immunological Assays

The specific and fairly stable reaction of antibody with antigen to form an antigen-antibody complex has encouraged a number of researchers to develop assays for the measurement of antigens and antibodies in specimens. The reaction takes place even at extremely low concentrations of the reactants which may include not only biological antigens such as nucleic acids, steroids, antibiotics, hormones, enzymes and microorganisms but also synthetic substances that are immunogenic. Some of the procedures that have been developed include: agglutination reactions with erythrocytes or latex beads, complement fixation, haemagglutination inhibition, immunodiffusion, serum neutralization, immunofluorescence, radioimmunoassay and enzyme-linked immunosorbant assay. The principles of specific binding of antibody to antigen is common in all these techniques, the differences lying in the means of detection of this immunoreaction. It is at the level of detection that the sensitivity, or ability to detect an immunoreaction, even at very low concentrations of the immunoreactants, is determined. Techniques such as immunofluorescence, radioimmunoassay, and serum neutralization are very sensitive but have inherent problems of lengthy incubations, costly equipment or use of radioisotopes that limit their applicability.

1.3 Antibody-Coated Protein A Bearing *Staphylococcus aureus* as an Immune Reagent

The attractive aspect of protein A is that it binds immunological molecules with high affinity, the principle molecule bound being IgG although other classes of immunoglobulins are bound to lesser, varying degrees (Goding, 1978). Protein A has been successfully used for indirect immunoprecipitation of immune complexes in radioimmunoassay and for molecular characterization of cell surface antigens (Natali, *et al.*, 1979).

Protein A is covalently linked to the peptidoglycan part of the cell wall of most strains of *Staphylococcus aureus* although it is produced in varying amounts by different strains. Among the best protein A producers is the *Staphylococcus aureus* Cowan I strain. The protein A is a single polypeptide chain of molecular weight 42,000, and has little or no carbohydrate content. It has three highly homologous, F_c-binding regions of which at least two are available for IgG binding via the F_c region of the gamma chain. Protein A is very heat stable and is resistant to many denaturing agents. There are somewhere in the order of 80,000 molecules per organism and it has been estimated that 100µl of packed bacteria will bind 0.8 - 1.5mg of IgG (Goding, 1978). Natali, *et al.*, (1979) found that after prolonged storage under a variety of conditions antibody-coated *Staphylococcus aureus* Cowan I strain maintained a high immunological reactivity.

On the basis of the above facts and observations it was thought that protein A bearing *Staphylococcus aureus* Cowan I strain may be a useful immunological reagent for detecting rotaviral antigens in faecal suspensions. A system was designed to use protein A bearing bacteria, coated with rabbit IgG specific against SA_{11} rotavirus. This was used as a solid phase immunological reagent that agglutinated into visible clumps in the presence of rotaviral antigen present in faecal specimens.

1.4 Radioimmunoassay (RIA)

Radiolabelled antigens and antibodies have been widely used in the study of a variety of biological problems. Berson and Yalow (1968) used radioactively labelled antigen in their study of hormones. Application of RIA techniques to serological diagnosis of viral diseases was slow. The development of techniques to radioactively label antibody and adaptation to solid phase and rapid micro assays made the system more applicable to viral diagnosis. Hunter and Greenwood (1962) developed a procedure for labelling protein with radioactive ^{131}I using chloramine T. Solid-phase assays, using either the attachment of virus or antibody to various solid support systems, were developed. Rosenthal, et al., (1972) developed a micro assay by fixing antigen to wells of a flexible polyvinyl microtitre plate. After reacting with specific antiviral antibody and then radio-labelled antibody, the wells were individually cut out and counted in a gamma spectrometer. The antigen can be fixed to the solid phase by physically drying the antigen on. Multiple washing steps do not remove the fixed antigen. The advantage of this system is its sensitivity when compared to complement fixation or serum neutralization tests. It is also a rapid, simple technique for which only small amounts of sample are needed and the results can be quantitated. The theoretical aspects of antigen-antibody reactions in micro-solid phase RIA have been investigated in an effort to maximize the specificity and sensitivity of the assay (Kalmakoff, et al., 1977; Maskill, 1978). This well characterized rapid and sensitive technique was first used for diagnosis of rotavirus antigen in faecal specimens by Kalica, et al., in 1977. They used a direct-sandwich assay where specific anti-rotavirus antibody was fixed onto microtitre wells, faecal suspensions were added and then after washing, ^{125}I-labelled anti-rotavirus IgG was added. The bound radioactivity was determined and indicated the amount of rotavirus antigen present in the specimens. The assay

took 3 to 4 days to perform and proved to be no greater in sensitivity than EM. Problems of false positive reactions were overcome by using a blocking test. The incubation times were later lowered so that their whole assay took no longer than 2 days to perform. The advantages that Kálica, et al., (1977) found for RIA over EM were that it was easier to perform, could be used to test small amounts of crude specimen, and mainly that it could be used to screen large numbers of samples in a single test in a relatively short time.

Watanabe, et al., (1978) also developed a solid-phase RIA for detecting rotavirus antigens and antibody. The solid phase was a paper disc rather than a microtitre well. This group found that RIA was less sensitive than EM. A suggested explanation for this result was that virions in faeces may be covered in antibody, hence if antigenic determinants of virions were already completely covered with antibody then RIA would not detect them whereas visualization by EM still would.

Middleton, et al., (1977) developed a solid phase RIA for the detection of rotavirus in the form of purified antigen or unpurified as in faecal specimens. The parameters of the assay were optimized to give high sensitivity and same day results. They found the system to be at least as sensitive as EM for faecal specimens and at least 100 times more sensitive for detection of purified virus antigen.

The main limitation of the RIA system is its use of a radioactive label. This means there is a necessary requirement for facilities to store and handle radioisotopes and expensive equipment for counting the radioactivity, which many laboratories are not able to afford.

For this reason we looked for a technique that would be as sensitive as RIA, would be rapid, able to handle large sample numbers in small volumes but would not need expensive equipment and could be performed in any diagnostic laboratory.

1.5 Enzyme-Linked Immunosorbant Assay (ELISA)

This section covers the development of the ELISA system, the principles involved, problems, and some of the applications of the system.

1.5.1 General Principles and Types of ELISA. The general principle behind this assay system is that antibody and antigen bind specifically by immunoreaction and that either of the immunoreactants can be labelled with an enzyme. The antigen-antibody complex can then be detected by addition of a substrate which reacts specifically with the enzyme and is usually detectable by a colour development. The development of solid phase assays where either antigen or antibody is adsorbed onto a plastic surface and the antigen-antibody complex forms on it, has meant easier handling of assay systems, particularly with washing steps and a decrease in non-specific, false positive reactions. A number of assay systems are outlined in Fig. 1. For the detection of specific pathogens in clinical specimens types 2 and 4 are most commonly used. In type 2 a specific antibody adsorbed to a solid phase is used to "capture" the desired antigen from the specimen suspension. After a washing step to remove any unbound material, a second antibody also specific against the antigen is allowed to react. After appropriate washing this second antibody is detected by adding an enzyme labelled antibody specific against the intermediary antibody. Any unbound labelled antibody is removed by washing and bound antibody is detected when substrate is added. In type 4, the clinical specimen is dried directly onto the solid phase without the use of a capture antibody. The rest of the assay system is the same as for type 2. For the detection of specific antibody, antigen can be adsorbed onto the solid phase and then sera, urine, milk, etc., can be screened for the presence of antibodies. The advantage of the type 2 sandwich assay over type 4 is that only the specific antigen desired from the clinical specimen is left bound to the antibody-solid phase and consequently a lot of other material that may cause

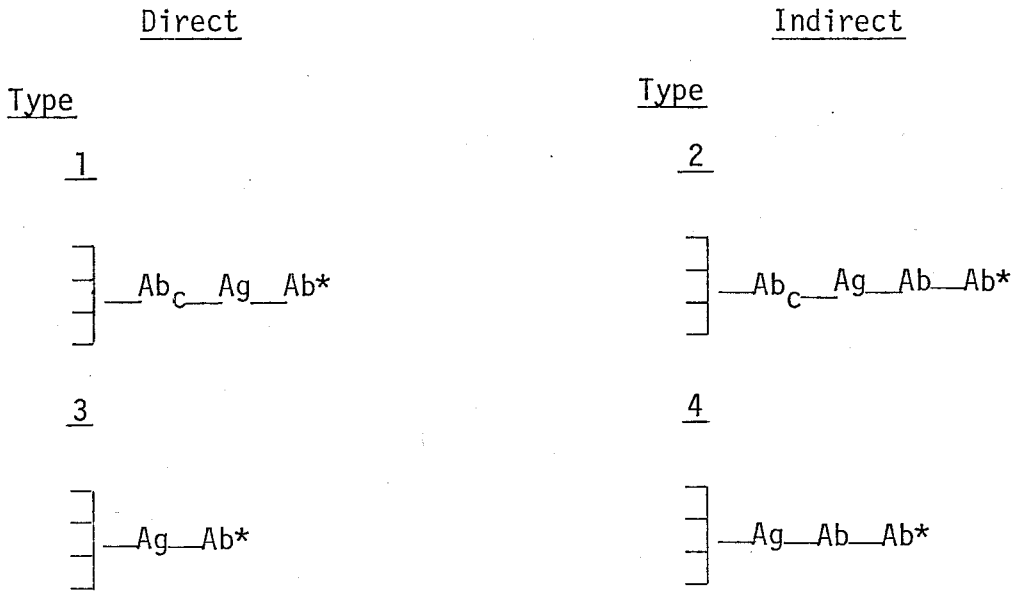

Fig. 1--ELISA Detection Systems.

1 Direct sandwich technique.

2 Indirect sandwich technique.

3 Direct technique.

4 Indirect technique.

solid phase.

Ab_c capture antibody.

Ag antigen

Ab antibody specific to antigen

Ab* enzyme labelled antibody

nonspecific binding of antibody or interference of the enzyme reaction is removed upon washing. The advantage of the indirect over the direct system is that, 1) one conjugate (antibody-enzyme) can be used for a variety of infectious agents, and 2) more than one labelled antibody molecule will bind to each intermediary antibody molecule resulting in an amplification step which means less antibody can be detected and hence making the assay system more sensitive.

1.5.2 Choice of Enzyme. Enzymes have proved to be suitable labels for detecting immune reactions for a number of reasons, some of which have already been mentioned. One of the main advantages is that because of their catalytic properties they act as amplifiers and so make the assay system very sensitive.

To be suitable as an enzyme label for immunoreactions an enzyme must meet certain requirements:

1) Available commercially at low cost and in high purity.

2) Has a high specific activity.

3) Is stable under assay and storage conditions.

4) Is soluble.

5) Measurement of activity is simple, sensitive, rapid and cheap.

6) Is absent from biological specimens.

7) Substrates, inhibitors and disturbing factors are not likely to be met in biological specimens being assayed.

8) The presence of reaction groups in the enzyme to allow linking to other molecules while retaining a substantial part of the enzymatic activity (Wisdom, 1976; Nakane, 1979; Schurrs and Van Weemen, 1977).

Obviously some enzymes are going to be more suited to certain types of assay than are others, depending on what type of biological specimens are being assayed and the method being used. Some of the enzymes that have been used as labels in immunoassays are:

1) Malate dehydrogenase from pig heart mitochondria.

2) Glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides.

3) Glucose oxidase from fungi.

4) Peroxidase from horseradish.

5) Acetylcholinesterase.

6) Alkaline phosphatase from calf intestinal mucosa and E. coli.

7) Glucoamylase from Rhizopus nivens.

8) Lysozyme from egg white.

9) β-Galactosidase from E. coli. (Wisdom, 1976)

The two most commonly used enzymes are alkaline phosphatase (AP) and horse-radish peroxidase (HRP). At a meeting "Enzyme Linked Immuno Specific Assay for Infectious Agents", (National Institute of Health, Bethesda, MD, USA, 1976) it was concluded that HRP and AP were the two enzymes of choice being about equal in performance and because of the brightness of the colour developed with chromagens, thus allowing more sensitive detection. HRP has three main advantages over AP:

1) It is unusually stable and can be stored for long periods without losing significant activity.

2) Purified HRP is much less expensive than AP.

3) The HRP reaction produces bright colour with the appropriate chromagens, permitting easy visual and colourimetric reading (Bullock and Walls, 1977).

Peroxidase activity is assayed using H_2O_2 (hydrogen acceptor) and a chromagen (hydrogen donor). Three chromagens that have been commonly used with peroxidase are: 2, 2-azino-di-(3-ethyl-benzthiazoline-6-sulphonate)-ABTS; 5-amino salacylic acid-5AS; and ortho-phenylenediamine-OPD. Saunders (1979) in a comparative study found OPD to be more sensitive than ABTS which was in turn more sensitive than 5AS. OPD gives a very sensitive reaction with HRP and H_2O_2, is easy to stop but is very unstable in light. ABTS is sensitive,

very stable but requires a carefully prepared stopping reagent. And lastly, 5AS is not sensitive, is unstable, is difficult to stop and hence is not recommended for use.

Once the choice of enzyme has been made and the type of assay to be used has been determined, the enzyme then needs to be conjugated with either the antigen or antibody.

1.5.3 Enzyme Labelling of Immunoglobulins. A number of cross linking reactions for enzymes to antibody or antigen have been developed. A suitable cross linking reaction should produce a good yield, a stable conjugation of enzyme and antibody or antigen, with adequate labelling and with minimal impairment of enzyme activity and immunoreactivity. As HRP is the enzyme of choice in most ELISA systems, methods for linking HRP to antibody or antigen have been outlined below.

A. One-step Gluteraldehyde. This method links through the free amino groups of the two interacting molecules. Conjugates are formed by mixing enzyme and antibody in the presence of gluteraldehyde. The resulting conjugates are often of high molecular weight and heterogeneous in nature. It was found when cross linking HRP with IgG that only 5% of the HRP conjugated, and there was large losses of antibody activity. Self-linkage of IgG was also extensive (Wisdom, 1976).

B. Two-step Gluteraldehyde. HRP when reacting with gluteraldehyde only reacts with one aldehyde group of the gluteraldehyde molecule, the second aldehyde group of the cross linker is unable to react with the same or other peroxidase molecules. This mechanism is basic to the two-step method as it minimizes self-coupling of the enzyme. The peroxidase is treated with gluteraldehyde, excess is removed, and the "activated" peroxidase is mixed with the antibody, allowing the free amino groups to react. Gluteraldehyde reacts with proteins chiefly at the ε-amino group of the lysine residues. The structure of the linkage is not clear, Schiff bases

are probably formed and stabilized by secondary reactions. Losses of antibody activity do occur but are not as great as with the one-step method. Incorporation of the enzyme into the conjugate does not appear to be large and may be less than for the one-step method (Wisdom, 1976).

C. Periodate. Horse-radish peroxidase has several oligosaccharide groups which can be oxidised to form aldehyde groups that can then react with amino groups; this is the basis of the method of linkage with antibody molecules. To prevent coupling of newly formed aldehyde groups on one molecule of HRP with ε-amino groups of another, two methods have been employed successfully. The first is to block the ε-amino groups with 1-fluoro-2,4-dinitrobenzene (FDNB) or secondly, to prevent self coupling by lowering the pH of the environment so that the majority of the ε-amino groups are unable to react with the aldehyde. The carbohydrate moiety of HRP is oxidised by sodium periodate to form aldehyde groups. Formation of the HRP aldehyde inhibits HRP activity, most of which is recovered upon conjugation with antibody. The "activated" HRP is then allowed to react with free amino groups of antibody molecules forming Schiff base linkages. The linkage is stabilised by being reduced with sodium borohydride. The efficiency of linkage is dependent on the amount of sodium periodate employed and the duration of oxidation. Approximately 70% HRP and 99% IgG can be incorporated into conjugate. One problem observed with this method is that "activated" enzyme molecules, because they have several oligosaccharide groups may cross link two or more IgG molecules resulting in conjugates of high molecular weight. HRP-IgG conjugate preparations by the periodate method are superior to preparations using the two gluteraldehyde methods in terms of their sensitivity (Wisdom, 1976; Nakane and Kawaoi, 1974; Schurrs and Van Weemen, 1977).

Methods of separation of bound and free labelled molecules should give complete separation, be non-disruptive, rapid, reproducible, simple,

cheap and not subject to nonspecific interference. Methods used have been separation by Sephadex G-200, ultrogel ACA-44, density gradient centrifugation and salt fractionation (Wisdom, 1976; Schurrs and Van Weemen, 1977).

The prepared enzyme conjugates can be very stable if stored appropriately. Freezing and thawing of conjugates forms aggregates. If, however, it is stored at 4^{o} or room temperature, HRP, AP and β-galactosidase conjugates have been found to lose little activity over periods of a year or more (Wisdom, 1976).

1.5.4 Factors Involved in the Accuracy and Specificity of ELISA Systems. The ELISA system is rapid and relatively easy and cheap to set up and run. However, the accuracy and sensitivity of the system is only as good as the reagents, materials and expertise involved. A number of factors can lower the sensitivity of the assay and may cause interference or false results. Some of the factors that need to be considered in setting up the system are the reagents being used, the equipment, conditions and length of incubations, washing procedures, and handling of specimens in order to eliminate interference or nonspecific binding.

The sensitivity and accuracy of the system is particularly dependent on the antigens and antibodies involved. For antigen detection the specificity of the antibodies needs to be high. An antiserum that contains antibodies, at a relatively high level, to a number of antigens is obviously going to lower the accuracy of the results obtained. Accuracy can often be obtained by using the antiserum at a high dilution so that only antibodies to the specific antigen(s) with which the animal has been immunized will still be present in significant amounts. The use of pre-immunization sera from the same animal as a control helps to detect any inaccuracy in the results. If the antiserum has to be used at a high dilution in order to dilute out unwanted antibody then the antibody required for detection of the specific antigen needs to be at very high levels. It is the hyperimmune

antibody level and its affinity for the particular antigen that affects the sensitivity of the system. In the indirect ELISA (types 2 and 4, Fig. 1) system the conjugated antibody also has a large effect on the accuracy and sensitivity of the assay. The conjugated antiserum must also be used at a relatively high dilution so that only antibodies specific against the intermediary antibody are present in significant amounts. The system would be very inaccurate if the conjugated antiserum contained high levels of antibodies to antigens in the specimen being assayed. Consequently there is a need to test conjugated antibody directly against the specimen as another control. The amount of enzyme conjugated to antibody in the conjugate preparation will also effect the sensitivity of the system. If there is a large amount of antibody that is free of enzyme this will prevent the binding of conjugated antibody and will consequently lower the assay sensitivity. Properties of the conjugated antibody that will effect the sensitivity of the system are: enzymatic activity, immunological activity, the amount of noncoupled enzyme present, the amount of noncoupled immunoglobulin, the number of enzyme molecules per molecule of immunoglobulin, the biochemical properties, and the performance of the conjugate in the particular type of assay used. The ELISA system is only as accurate and sensitive as the specificity and concentration of antibody in the antisera used. Careful immunization and screening of antibodies produced is essential for the standardization and interpretation of the assay system.

The materials and methodology used will also have a secondary effect on the accuracy of the assay. A number of solid phases have been evaluated for use in the ELISA system. These include glass rods, various types of plastic rods, tubes, trays, spheres and cellulose discs (Saunders, 1979). For micro systems the commercially available microtitre trays have been widely used. The advantage of these trays is the small volumes that can be used for a comparatively large surface area and the ease of handling large

numbers of specimens. Bullock and Walls (1977) conducted a comparative study of seven types of microtitration trays and their usefulness as absorbants for antigen in ELISA. They compared two polystyrene trays with flat-bottomed wells, two polystyrene U trays, one polyvinyl chloride U tray, one polyvinyl chloride V tray and one polyvinyl chloride flat tray. Of those tested, the polyvinyl chloride trays gave results visually superior to polystyrene trays. They found no marked variation in serum titres between any of the trays but the flexibility of the polyvinyl chloride trays did cause some handling difficulties. Bidwell, et al., (1977) compared the binding efficiency for antibody of a polystyrene tray, a polyvinyl chloride tray and a polystyrene tray specially treated for tissue culture. They found that the tissue culture trays were quite useless as they had a high nonspecific uptake of material that masked the differences between positive and negative results. The polystyrene and polyvinyl chloride trays gave similar results to each other. In a comparison of adsorption of ^{125}I labelled rabbit IgG to different plastics (cellulose nitrate, polyallomer, polystyrene and polyvinyl chloride)Hermann and Collins (1976) found that polyvinyl chloride was the most efficient absorber of IgG when in low concentration, absorbing up to 96.9% of the radioactivity added in 18 hours at a protein concentration of 1µg/ml. After one hour of incubation it had absorbed 69.4%. At higher concentrations of protein the efficiency of absorption was much reduced -- 100µg/ml only absorbed 10.2% and 10µg/ml - 53.4% after 18 hours. For reading of results in commercially produced ELISA readers, flat-welled polystyrene trays are now produced with special optical properties. For assays where a quantitative answer is not needed but rather a straight positive or negative result, reading of results by eye is adequate, then polyvinyl chloride trays are most suitable.

Once a method for efficient binding of antigen or antibody to the solid phase has been determined the next factor that may effect the accuracy

of the assay is nonspecific binding of antibody to the specimen. In an assay where antigen has been adsorbed directly to the solid phase this nonspecific binding may occur via two mechanisms. Firstly, antibody may itself adsorb to free sites on the plastic solid phase or secondly, antibody may bind to the biological material by other than immunoreactive means. Such nonspecific binding can be monitored by including appropriate controls. The use of preimmunization serum as a control will indicate whether the intermediary antibody, in an indirect assay, is binding nonspecifically. Nonspecific binding of the conjugated antibody can be checked by reacting conjugate directly with the specimen without the intermediary antibody. The nonspecific binding may be greatly reduced by either blocking available sites on the solid phase or by creating conditions that would minimise such binding. Blocking of the solid phase can either be achieved by incorporating an incubation step with protein such as bovine serum albumin or by adding foetal calf serum (FCS) to the antibody dilutions. The principle in the second method is that the non-immunoreactive protein of the FCS, being in a much higher concentration than antibodies of the specific antiserum, will adsorb more readily to any available sites remaining on the solid phase. The alternative method to blocking sites is to create conditions such that nonspecific binding is less likely to occur. The adsorption of antigens to the solid phase is based on hydrophobic interactions between the plastic and protein. Such interactions, once they have occurred are essentially irreversible. Non-ionic detergents, however, will prevent this kind of interaction but will not reverse it. Consequently, once the desired antigen has been allowed to adsorb to the solid phase, subsequent use of a non-ionic detergent in the diluent of sera and conjugate will largely prevent nonspecific binding of the immunoreactants to the solid phase but will not interfere with the specific antigen-antibody reaction (Engvall and Ruoslahti, 1979). Saunders (1979) found that use of

a buffered, high molar salt solution, containing 0.5M NaCl, pH 8.0 and 0.5% Tween 80, as diluent for the intermediary antibody and conjugate prevented much of the nonspecific binding without hindering the specific reaction of positive sera. Aggregated immunoglobulin binds very quickly to plastic solid phases, consequently test sera need to be treated as carefully as possible to reduce aggregation of Ig. Saunders (1979) suggested that positive sera that has been frozen and thawed repeatedly should be given a quick centrifuge in order to remove any aggregates.

Rheumatoid factor has also been found to be a possible source of nonspecific binding particularly in sandwich assays (types 1 and 2) for both RIA and ELISA systems (Wisdom, 1976). Interference by the rheumatoid factor can largely be avoided by the addition of aggregated IgG to the sample prior to or during the first steps of the assay (Schurrs and Van Weemen, 1977).

Other factors that may cause nonspecific interference of the immunoreactants are background enzyme activity in the sample tested or inhibitors of the enzyme label present in the sample (Wisdom, 1976).

Overall the ELISA system has proved to be a specific and highly sensitive method for the identification of a wide range of antigens and antibodies. Compared to RIA it is generally lower in sensitivity and may be more susceptible to interference by nonspecific factors. ELISA cannot be fully quantitated without the use of expensive equipment to measure rates of enzyme activity and consequently endpoints of titrations tend to be harder to establish than for RIA. However its simplicity and cheapness to set up and run, plus its specificity, sensitivity and rapidity have established it as a technique with many applications, particularly in the field of diagnostic virology (Wisdom, 1976).

Schurrs and Van Weemen (1977) compared ELISA, RIA and Immunofluorescence (IF). Their results are summarized in Table 1.

TABLE 1

Comparison of ELISA, RIA and IF (Schurrs and Van Weemen, 1977)

	ELISA	RIA	IF
Sensitivity	High	Very High	High
Specificity	Mainly dependent on the quality of antisera		
Precision	Good	Good	Low
Assay Time	Hours	Hours	Hours
Legal Requirements	None	Licence for handling radioactive material	None
Equipment	Photometer	Isotope Counter	Fluorescence Microscope
Automation Potential	High	High	Low
Required Expertise	Medium	High	High
Reagent Stability	High	Low	Variable

A number of aspects of ELISA systems require further investigation:

1) The standardization of conjugate preparations (Nakane, 1979).

2) Automation of the assay system to allow for screening of a greater number of specimens in a short time period, and

3) Quantitation of assay results to increase the precision of results.

ELISA was soon noted to have potential as a diagnostic assay system that would give rapid results. Its application to diagnosis of viral diseases was particularly noted where many of the techniques previously used were either not particularly rapid or very sensitive. The first reported use of ELISA in virology was for the detection and measurement of rubella antibody (Voller and Bidwell) in 1975. In 1975 Voller, et al., adapted the ELISA system for the detection of plant viruses using a double-sandwich technique and AP in the detection system. Also in 1977 Wolters, et al., used ELISA for the detection of hepatitis B antigen in human sera and in 1977, Mathiesen, et al., for the detection of hepatitis A antigen in faeces.

By 1977 rotavirus was recognized as the major cause of infantile gastroenteritis epidemics but existing techniques for identification by EM, complement fixation and RIA were time consuming or demanded expensive equipment. Consequently the sensitivity, rapidity, cheapness and simplicity of ELISA meant it was an attractive technique for rotavirus identification. Yolken, et al., in August 1977, published the first report of ELISA being used for rotavirus antigen detection from stool suspensions. Like Voller, et al., (1975) they used a double-sandwich assay (type 1, Fig. 1) with the enzyme AP. They stated that "ELISA provides an accurate and rapid means for diagnosis of human reo-virus like agent (HRVLA) infection; we found it to be both as sensitive and as efficient as RIA and EM for detection of HRVLA." Later in the same year, Ellens and De Leeuw (1977) reported

the use of ELISA for the diagnosis of rotavirus infections in calves. They also used the double-sandwich technique but used HRP as their detector enzyme. Using a microtitre procedure they found the assay particularly suitable to large scale testing of field samples. A detection limit of 1ng/ml of virus protein showed the sensitivity of the system to be about 100 times higher than that of immunoosmoelectrophoresis. Using an ELISA blocking test, Yolken, et al., (1978) were able to distinguish rotavirus isolates from different host species and the existence of human rotavirus groups with different blocking patterns which presumably represented two different serogroups.

The sensitivity and simplicity of the ELISA system for handling large numbers of specimens and its applicability to small laboratories made this technique very attractive for our own studies and as a technique that could be used in human and veterinary diagnostic laboratories for rotavirus detection. In this section is reported the development and use of four techniques for rotavirus identification: EM, RIA, staphylococcal protein A agglutination, and ELISA. The ELISA system proved to be the most adaptable method while retaining high sensitivity and specificity.

2. MATERIALS AND METHODS

2.1 Collection of Specimens

Faecal specimens were collected from a number of sources and from a number of different host species. Human faecal specimens were collected as part of an ongoing survey of hospitalized infantile gastroenteritis patients from the Otago and Southland regions. These specimens arrived undiluted, usually in specimen jars, but during gastroenteritis outbreaks, infants napkins were received from which the faecal material had to be removed. Specimens were stored, undiluted at -20^{o}C until they could be assayed for rotavirus. After screening of specimens for rotavirus, positive specimens were stored at -20^{o}C and used for further analysis by either polyacrylamide gel electrophoresis or cDNA-RNA hybridization experiments. Some negative specimens were kept to be used as controls in further experiments.

Animal faecal specimens were collected largely from the Dunedin urban area or the Taieri Plain, a rural area southwest of Dunedin. Some specimens were received from the Ministry of Agriculture and Fisheries, Animal Health laboratories at Invermay, near Dunedin, and Wallaceville. The specimens from the rural area were from piglets, calves and foals. The majority of the specimens were collected from particular farms which were frequently visited during breeding seasons. The general condition of the livestock was noted and any scouring problems recorded. Contact with these properties was maintained for a period of three years.

Dog and cat specimens were collected from the Dunedin urban area through frequent visits to local cat breeders and the SPCA boarding kennels. Local veterinary surgeons were also visited and specimens were either received direct from them or information of animals with diarrhoea was received and contact then made with the particular owners.

Human sera was collected as part of the laboratory course for undergraduate students of Microbiology at the University of Otago. Sera was collected from third-year students for two consecutive years. The serum was diluted 1:2 in glycerol and stored at -20 °C until assayed. The ages of these students ranged from 20 to 23 years and as far as was known none were parents of young children.

2.2 Electron Microscopy

2.2.1 Faecal Specimens. Visualization of rotavirus particles in faecal specimens was made possible by making a 10-20% suspension of the faecal material in either distilled water or sterile PBS (pH 7.4). A drop of this suspension was dried onto a formvar coated copper grid and negatively stained with 1% phosphotungistic acid. The grid was then examined in either an Hitachi type HU-114 or Seimens ELMISKOP-102 electron microscopes at an instrument magnification of 20,000 x or greater.

Concentration of virus particles for EM visualization was achieved by centrifuging the faecal suspension at 10,000 g for 10 minutes to remove the large organic debris, then spun at 40,000 g for 90 minutes to pellet the virus. The pellet was then resuspended in either distilled water or PBS and a drop (approximately 1 µl volume) dried onto a formvar grid and treated as above.

2.2.2 Tissue Culture Grown Rotavirus. Cells infected with rotavirus were frozen and thawed three times to burst the cells and release any virus particles. The suspension of cell debris and virus particles was spun at 10,000 g for 10 minutes to remove the larger debris. The virus particles were then concentrated by pelleting at 40,000 g for 90 minutes. The pellet was resuspended in distilled water. The virus was then repelleted at 40,000 g for 90 minutes to remove any remaining tissue culture fluid which may interfere with staining procedures. The virus pellet was

resuspended in a drop of distilled water and dried onto a formvar grid negatively stained with 1% phosphotungistic acid and examined by electron microscopy.

2.2.3 Estimation of Virus Concentration. The copper grids used to examine both faecal and tissue culture specimens were estimated to contain approximately 250 squares per grid. Approximately 1 µl of virus suspension was dried onto a copper grid for examination. By counting the number of particles observed in a number of squares over a wide area of the grid and multiplying the average by 250, an estimate of the number of particles in 1 µl was made. By further multiplying this figure by 10^3 an estimate of the number of virus particles per ml was reached.

2.3 Staphylococcal Protein A

2.3.1 Preparation of Antibody Coated Staphylococcus aureus.

Staphylococcus aureus strain Cowan I (NCTC 8530) was grown in nutrient broth overnight at 37 °C. The fresh culture was spun at 7-8,000 g and washed in PBS, pH 7.4, and suspended in 0.5% formaldehyde at room temperature for 3 hours. The cells were then washed four times in PBS and adjusted to 10% (v/v). This preparation could be stored at -20 °C for at least a year and retain protein A activity.

The agglutinability of the preparation is improved by flash heating to destroy staphylococcal enzymes. The preparation was poured into a sterile, preheated petri dish floating in an 80 °C water bath. After 3-5 seconds the dish was withdrawn and placed on ice to cool rapidly. This preparation will remain stable for a number of months at 4 °C and will absorb up to 2 mg of globulin per ml of a 10% suspension.

Antibody coated staphylococcus was prepared by mixing 3 volumes of a 10% suspension of staphylococcal cells with 1 volume of antiserum diluted in PBS. After 60 minutes incubation at 4 °C the bacteria were washed twice

with buffer and resuspended in buffer to a 10% suspension (v/v). Antibody coated staphylococcus were fixed with gluteraldehyde by mixing 1 volume of washed antibody coated bacteria with 10 volumes of a 0.05% gluteraldehyde aqueous solution and incubated for one hour at 4 °C. The bacteria were then washed extensively with buffer before use in the binding assay.

2.3.2 Detection of Rotavirus Antigen. Staphylococcal bacteria, coated with specific rabbit anti-SA_{11} rotavirus were diluted in PBS to a final concentration of 5 x 10^9 cells per ml. The faecal specimen was suspended in PBS and spun at 7,000 g for 10 minutes. The supernatant was reacted against the agglutination preparation by mixing one drop of each on a clean glass microscope slide. The suspension was allowed to react for 5 to 15 minutes before being examined for agglutination.

Staphylococcal bacteria coated with normal rabbit sera was reacted with the faecal preparation in the same way as a negative control for non-specific agglutination.

2.4 Radioimmunoassay (RIA)

An indirect RIA (type 4, Fig. 1) was used to detect rotavirus antigen in faecal suspensions. There are three stages however that had to be completed before the assay for detection of specific antigens could be used. Firstly, the preparation of specific antisera; secondly, the radiolabelling of the detection antibody; and thirdly, the standardization of the antisera used in the detection system.

2.4.1 Preparation of Specific Antisera. Antibodies were induced in rabbits against a) Simian (SA_{11}) rotavirus, and b) reovirus type 3. Antibodies against rabbit IgG were prepared in sheep.

a) Rabbit anti-SA_{11} Rotavirus. A rabbit was bled prior to immunization as a preiummunization control of antibody levels already present

in the animal. The initial immunization was with a 1 ml mixture containing 100µg of purified, tissue culture grown, SA_{11} rotavirus in an equal volume of complete freunds adjuvant (CFA) administered intraperitoneally. The rabbit was given two boosts of antigen of approximately 100µg of SA_{11} virus each. Immediately prior to each boost the rabbit was bled for testing of antibody levels. The rabbit was eventually bled out by cardiac puncture 42 days after the initial inoculation and 11 days after the final boost. The collected sera was stored in 50% glycerol at -20°C.

b) Rabbit anti-Reovirus Type 3. A preimmunization bleed was made and then the rabbit was inoculated with 260µg of purified reovirus in CFA, intraperitoneally in 2 sites. After 12 days the rabbit was boosted with 100µg of reovirus and then again 35 days later. The rabbit was bled prior to each boost. The rabbit was bled out by cardiac puncture 16 days after the last boost and the collected sera was stored in 50% glycerol at -20°C.

c) Sheep anti-Rabbit IgG. A sheep was inoculated intraperitoneally with 5mg of rabbit IgG in an equal volume of CFA. The rabbit IgG was purified by ammonium sulphate precipitation. Sheep antibodies were boosted by administration of further rabbit IgG, 500µg each time, 70 and 88 days after the initial inoculation. The sheep was bled out 50 days after the final boost and the sera was stored in 50% glycerol at -20°C.

2.4.2 Labelling of Immunoglobulin with ^{125}I. To one millicurie of ^{125}I were added 25µl of 0.25M phosphate buffer, pH 7.5. Then 100µl of immunoglobulin (1 to 2.5mg) and 20µl of freshly made chloramine T were added and gently vortexed for 90 seconds. 100µl of sodium metabisulphate was added and vortexed for 60 seconds. The mixture was left in a fume cupboard for 3 minutes to react. The free ^{125}I was separated from the labelled antibody by passing the reacting mix down a sephadex G50 column and collecting the fractions. Fractions were counted and the tubes containing

the labelled antibody were pooled. The antibody was ammonium sulphate precipitated and resuspended in 1ml of PBS/glycerol and stored at -20°C. Specific activity was determined by protein estimation and the radioactive count for 10µl of the final preparation.

2.4.3 Standardization of ^{125}I Labelled Sheep Anti-Rabbit IgG. The dilution of ^{125}I labelled sheep anti-rabbit IgG (SXR*) to be used in antigen detection assays was determined by titrating 4 known concentrations of SXR* against dilutions of rabbit IgG. Doubling dilutions of rabbit IgG, from 2µg/25µl to 1ng/25µl were dried onto microtitre wells, fixed with methanol (4°C) for 10 minutes and washed 6 times in PBS. The wells were blocked with 0.1% gelatin in PBS for 2 hours at room temperature and washed 6 times in PBS. The 4 concentrations of SXR* (0.4, 0.2, 0.1, and 0.05µg/25µl) diluted in 1% FCS-PBS were added and incubated for 4 hours at room temperature. The wells were extensively washed in PBS and counted. Nonspecific binding of the SXR* was checked by running a similar assay in parallel with the rabbit IgG dilutions being replaced with bovine gamma globulin (BGG).

2.4.4 Antigen Detection System. For detection of rotaviral antigen in faecal specimens a 10-20% suspension of faeces was made in PBS. 25µl of this suspension was added to a microtitre well and dried. The antigen was fixed with methanol at 4°C for 10 minutes. The wells were then washed 6 times in PBS. Rabbit anti-SA_{11} serum diluted in 1% FCS-PBS was then added (25µl) and incubated for 2 hours at room temperature. After washing 6 times in PBS the appropriate dilution of SXR* in 1% FCS-PBS was added and incubated for 4 hours at room temperature. After extensive washing the wells were counted. Controls used to check nonspecific binding of the two antibodies were normal rabbit serum and SXR* reacted directly against the faecal antigen with no intermediary rabbit antisera. A faecal suspension known to be positive for rotavirus and one known to be negative were included in specimen screens.

A modification that was used initially was to use a capture antibody as for type 2 (Fig. 1). The modification in technique was to coat the microtitre wells with goat anti-SA_{11} antiserum by incubating for 4 hours at 37°C and then fixing with methanol at 4°C for 10 minutes. The wells were then blocked with 0.1% gelatin-PBS for two hours at room temperature. After washing in PBS the faecal suspension was added to the wells and incubated for 2 hours at room temperature. The procedure then continued the same as for above except the methanol step was left out after the faecal antigen incubation. This method proved to be no more sensitive than without the capture antibody and was more time consuming.

A modification that was later employed was the addition of Tween 20 (0.05%) in PBS for the washing steps to cut down nonspecific binding.

2.5 ELISA

The assay system for detection of rotavirus antigen and antibodies evolved over the period of the study with modifications bringing improvements in specificity and sensitivity.

2.5.1 Conjugation of Enzyme to Antibody. The method most used for the conjugation of horse-radish peroxidase (HRP) to immunoglobulin G (IgG) was that of Nakane and Kawaoi (1974). The conjugation reaction involves the linking of the carbohydrate moiety of HRP and the amino groups of IgG. HRP has several oligosaccharide groups, and their oxidation to aldehyde groups, that can then react with the amino groups of IgG, is the basis of this method of linkage. The peroxidase was first treated with 1-fluoro-2,4-dinitrobenzene (FDNB) to block its free amino groups and prevent self linkage. The saccharides were oxidized with sodium periodate and the "activated" HRP was then allowed to react with the free amino groups of IgG. Schiff bases formed were then reduced with sodium borohydride to give stable linkages.

Specifically the method was to dissolve 5 mg HRP in 1 ml of 0.3 M sodium bicarbonate, pH 8.1. 100 µl of FDNB in ethanol was added and stirred for 30 minutes at room temeprature. The oligosaccharides were oxidised by adding 1 ml of 0.08 M sodium periodate and incubating for 30 minutes at room temperature. The reaction was stopped by the addition of 1 ml of 0.16 M ethylene glycol for 1 hour at room temperature. The HRP was then dialysed overnight against 0.01 M sodium carbonate, pH 9.5. The IgG (5-10 mg) was added to the 3 ml of HRP-benzene solution and allowed to react for 2 hours at room temperature. The mix was then cooled to 4 °C and the linkages stabilized by the addition of 5 mg of sodium borohydride and incubated for 2 hours on ice. Two drops of acetone were added and the mix left for 1 hour on ice. The reaction mixture was then dialysed overnight against 0.015 M PBS. Free HRP was separated from conjugated enzyme by passing the preparation down to G100 or 200 sephadex column. Fractions collected were read at 280 and 403 nm. An alternative method for separation was by adding an equal volume of saturated ammonium sulphate solution and incubating for 20 minutes to allow the IgG to precipitate from solution. IgG-HRP was pelleted at 10,000 g for 15 minutes, washed twice with 50% saturated ammonium sulphate and then dialysed against 0.01 M PBS.

The molar ratio of HRP to total protein as determined by the 403 to 280 nm readings gives an indication of the efficiency of linkage. Pure HRP gives an R_z value of 3. Peroxidase labelled IgG was stored at -20 °C in 50% FCS-PBS.

An alternative method used for conjugation was that of Nakane (1978). The method is essentially the same as for above except amino groups on the HRP molecule are not blocked by the addition of FDNB but are rendered unreactive by holding the pH at 4.0 during oxidation of the oligosaccharides and then raised to pH 9.5 to immediately react with the IgG.

HRP (4 mg) is dissolved in 1 ml of distilled water and 200 µl of freshly made 0.1 M sodium periodate. The solution is stirred for 20 minutes at room

temperature. The resulting HRP-aldehyde is dialysed against 1mM sodium acetate, pH 4.0, overnight. Meanwhile 8mg of IgG is dissolved in 1ml of 0.01M sodium carbonate, pH 9.5 and dialysed overnight in the same buffer. To the HRP-aldehyde 20µl of sodium carbonate, pH 9.5, is added and immediately the dialysed IgG is added. The reaction mixture is stirred for 2 hours at room temperature. Freshly made sodium borohydride (4mg/ml H_2O) is added at a volume of 0.1ml and left at 4°C for 2 hours. The conjugated IgG-HRP is separated from free HRP by passing the mixture down a column of sephadex G-100. The absorbance of the fractions is read at 280 and 403nm. The IgG-HRP fractions are pooled, the R_z value determined, and the conjugate stored at -20°C in 50% FCS-PBS.

Conjugation of HRP to IgG was made for sheep anti-rabbit, sheep anti-mouse, sheep anti-human, rabbit anti-cat, rabbit anti-dog and sheep anti-human IgA. R_z values were determined for each conjugate and all were titrated against the specific immunogen to determine the dilution of conjugate to be used in antigen and antibody detection assays.

2.5.2 Standardization of Immunoglobulin and HRP Conjugates. The efficiency of the antibody-enzyme conjugations was determined from the R_z values for each fraction of the preparation as it passed through the sephadex column. It is known that pure HRP has an R_z value of 403/280nm reading of 3. An estimate of the total protein can be made by taking a spectrophotometer reading at 280nm and dividing it by 1.8. For IgG-HRP conjugates an estimate of the unlabelled antibody or non-peroxidase protein in a conjugate preparation can be made by:

$$\text{non-peroxidase protein (mg/ml)} = \frac{\text{total protein - peroxidase protein}}{1.8}$$

$$\text{eqn. 1} \qquad = \frac{(280\text{nm reading}) - (403\text{nm reading} \times 0.33)}{1.8}$$

[the peroxidase protein 280nm reading will be equivalent to the (403nm x 0.33) because pure HRP has an R_z reading of 403/280 = 3/1, i.e., 280 = 403/3]

The total protein concentration of the IgG-HRP preparation is calculated by:

eqn. 2 $$\text{Total protein (mg/ml)} = \frac{\text{reading at 280nm}}{1.8}$$

By subtracting result 1 from 2 an estimate of the peroxidase protein in the preparation is made.

eqn. 3 peroxidase protein mg/ml = (2) - (1)

To then determine the molar ratio of enzyme to antibody the result of eqn. 1 needs to be divided by the molecular weight of IgG and the result of eqn. 3 by the molecular weight of HRP.

IgG = 150,000MW

HRP = 40,000MW

i.e., rabbit and cat IgG-HRP the R_z of 403/280 = 0.84/108

i.e., eqn. 1 = 0.44

2 = 0.60

therefore 3 = 0.16

Molar rations of enzyme to antibody:

$$= \frac{0.16}{40,000} : \frac{0.44}{150,000}$$

$$= 4 \times 10^{-6} : 2.93 \times 10^{-6}$$

i.e., 1.4 enzyme molecules to every antibody molecule.

2.5.3 Rotavirus Antigen Detection. The method used for the detection of rotaviral antigen in faeces from human, calf, foal, pig, dog and cat hosts as well as for tissue culture grown rotavirus, evolved over the

period of the study with a number of minor modifications. Essentially the solid-phase indirect method (type 4, Fig. 1) remained the same, modifications were to decrease nonspecific binding and involved changes in washing procedures, diluents and inclusion of various controls. The method routinely used over the last year of the study is outlined below.

Specimens for screening were prepared in the same way as for RIA (see 2.4.4). Antigen dilutions (50µl volumes) were added to microtitre wells and dried at 37°C overnight or by using a hair dryer. Antigen was fixed to the solid phase by flooding the wells with methanol at 4°C for 10 minutes. Wells were washed in PBS - 0.05% Tween 20, 6 times. Rabbit anti-SA_{11} sera, diluted 1/1600 in PBS-0.05% Tween 20-0.5M NaCl was added to the wells (50µl) and incubated at 37°C for 2 hours. Wells were washed as above. Sheep anti-rabbit-HRP conjugate diluted 1/500 in 10% FCS-PBS-0.05% Tween 20-0.5M NaCl was added to the wells (50µl) and incubated for 2 hours at 37°C. The microtitre wells were again washed as above and 100µl per well of freshly prepared substrate (see Appendix I) added to the wells and incubated for 15 minutes at room temperature in the dark. Controls included in all assays for antigen detection were for nonspecific binding of rabbit sera and sheep anti-rabbit-HRP to the antigen. To check for nonspecific binding of rabbit sera, preimmunization rabbit sera was reacted against antigen, and for sheep anti-rabbit-HRP serum, it was reacted directly with antigen missing out the intermediary antibody step.

The same preparations of antisera were used throughout the period of the faecal antigen survey for human and animal rotavirus. Preimmune rabbit sera was replaced by rabbit anti-reovirus sera when stocks were used. The rabbit anti-reovirus was screened against a number of known positive and negative specimens at an equivalent dilution to the preimmunization sera.

Results were read by eye and scored from 0 to 4 with increasing intensity of colour. A score of greater than 2 was normally taken as a positive result as long as control wells scored 1 or less.

2.5.4 Rotavirus Antibody Detection. Essentially the method for detection of human antibodies to rotavirus was the same as that for antigen detection except that the antigen was tissue culture grown SA_{11} rotavirus and the intermediary antibody came from human hosts. The detection antibody was sheep anti-human-HRP conjugate. Uninfected tissue culture cells were used as control antigen to check against nonspecific binding of the intermediary human sera.

Simian rotavirus (SA_{11}) was grown in BSC_1 tissue culture cells by the method of Clark (1979). Virus was spun out of tissue culture media at 25,000 rpm for 90 minutes, washed and resuspended in PBS. Uninfected cellular antigen was treated in the same way.

SA_{11} infected cell dilution (50µl volume) was dried onto the microtitre wells. Dilutions of human sera (50µl) in PBS-0.05% Tween 20-0.5M NaCl were incubated against the virus antigen for 2 hours at 37°C and washed six times in PBS-0.05% Tween 20. Detector antibody, sheep anti-human-HRP (50µl) was added to each well and incubated for 2 hours at 37°C. Again the wells were washed as above and fresh substrate, 100µl per well, was added and incubated for 15 minutes at room temperature in the dark.

Results were read by eye and scored as for antigen detection. Any positive human sera were further assayed by making doubling dilutions of the sera and reacting against SA_{11} rotavirus infected cells as above to determine the antibody titre of the sera.

3. RESULTS

3.1 Electron Microscopy

Examination of human and animal faecal specimens for rotavirus particles by EM proved to be a valuable technique. The virus is morphologically very distinctive when viewed at approximately 120,000 x's magnification (Fig. 2--SA_{11} rotavirus). Rotavirus has a distinctive wheel-like appearance. The virus has a double-shelled capsid but often the outer capsid appears to be missing. A rotavirus particle showing the outer shell is evident in Figure 2. In many particles the negative stain, 1% phosphotungstic acid, penetrates the core and causes the appearance of particles with dark centres and a lighter "halo" which is caused by the outer shells not being stained.

EM proved to be more useful as a confirmationary test rather than for initial screening of specimens. This was largely due to the amount of time needed to prepare and examine each sample which was in the order of at least 15 minutes. The screening of a large number of specimens collected on an almost daily basis made EM examination extremely time consuming during peak periods of an outbreak. Although no direct comparisons were made, it appeared that EM was not as sensitive a technique as ELISA and PAGE. Many specimens that were classified as rotavirus negative by EM in fact proved to be positive by ELISA and PAGE. Likewise a number of specimens that were classified as rotavirus positive by ELISA were not able to be confirmed by EM.

It was estimated that one square of an EM grid was $0.028mm^2$ in area and that an EM grid was $7.07mm^2$ in total area. Therefore, there are approximately 250 squares per grid. Approximately 1µl of specimen suspension is dried onto a grid. From these parameters it can be estimated that the number of virus particles per ml of specimen suspension is $(250\ X) \times 10^3$ where X = the number of particles observed per square. Faecal samples

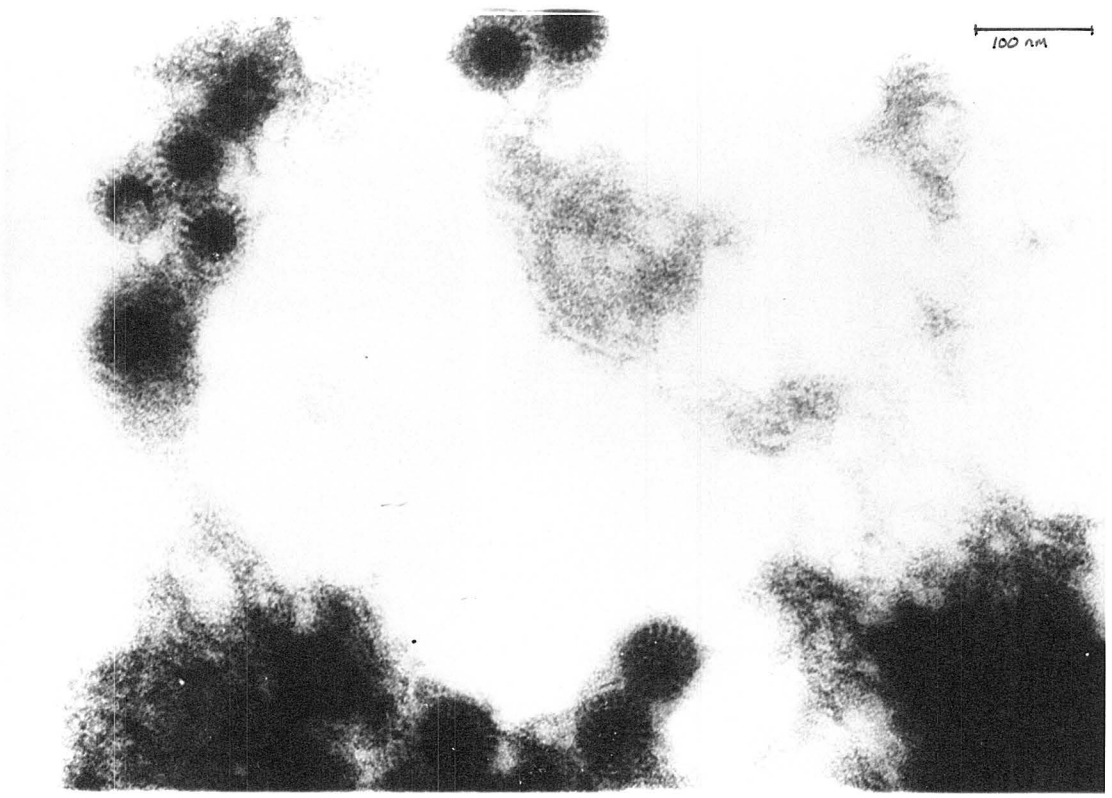

Fig. 2--Electron Micrograph of SA_{11} Rotavirus.

were diluted 1/20 for EM, therefore 20(250 χ) x 10^3 particles per ml would be present in the original sample.

In a suspension where only one particle was observed per square there would be an estimated 5 x 10^6 particles per ml in the original faecal specimen. Therefore, a specimen that had less than 1 x 10^6 particles per ml would be difficult to detect by EM as less that 1 in 5 squares would show rotavirus particles.

In tissue culture grown SA_{11} rotavirus, 150 particles were observed per square, i.e., in the semipurified suspension there were approximately 3.75 x 10^7 particles per ml. This was a greater number observed than for any of the faecal specimens examined.

Once the ELISA system had been developed and PAGE was used for detecting dsRNA, EM was used only as a confirmationary test rather than as a primary diagnostic technique. Specimens that were marginally positive by ELISA were further tested either by screening for dsRNA by PAGE or by looking for whole virus particles by EM.

Positive specimens from new host species were also checked by EM to confirm rotavirus particles. Cat rotavirus having been detected by ELISA was observed by EM in mid 1979 and later confirmed by PAGE. The EM revealed only few virus particles, a clump of which is shown in Fig. 3. Dog rotavirus was detected by ELISA in mid 1980 from a litter of greyhound pups. Virus particles (Fig. 4) were observed in a number of the specimens examined. Rotavirus was also observed by EM in human and calf specimens and in tissue culture grown preparations.

3.2 Staphylococcal Protein A

Staphylococcus aureus strain Cowan I (NCTC 8530) was grown as described in Materials and Methods (2.3.1), formaldehyde and heat killed, and adsorbed with specific antisera. Rabbit anti-SA_{11} rotavirus IgG, prepared

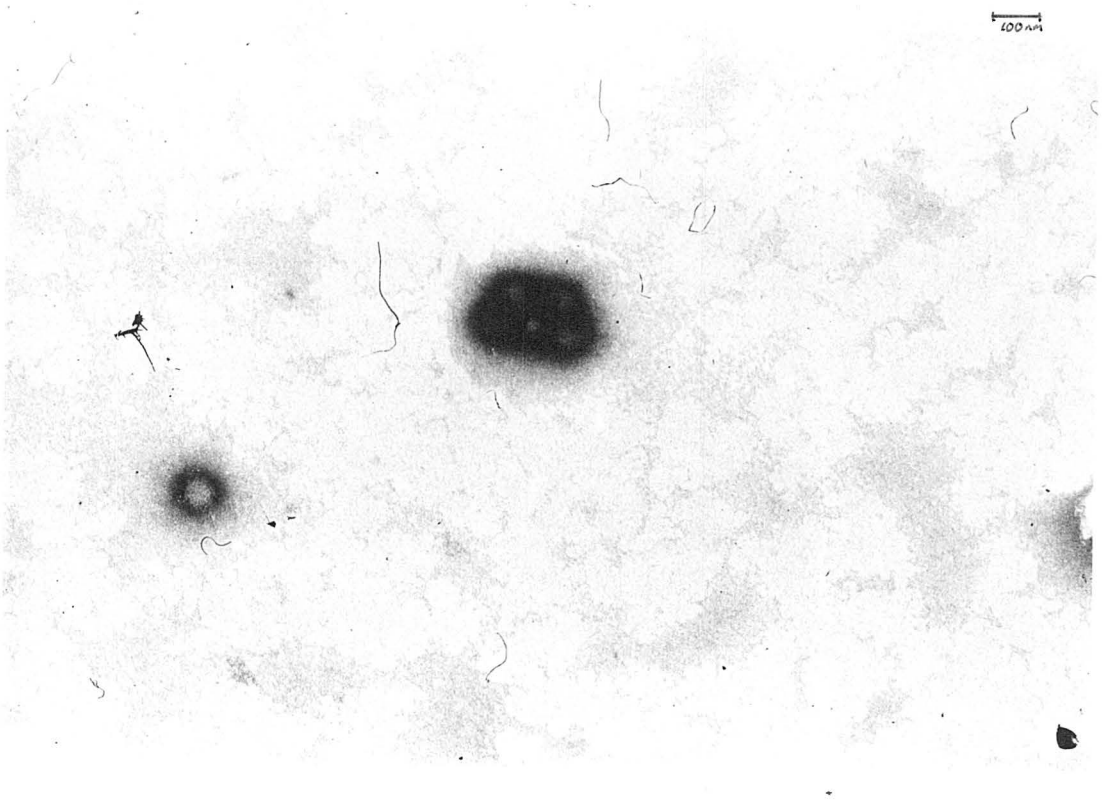

Fig. 3--Electron Micrograph of Cat Rotavirus.

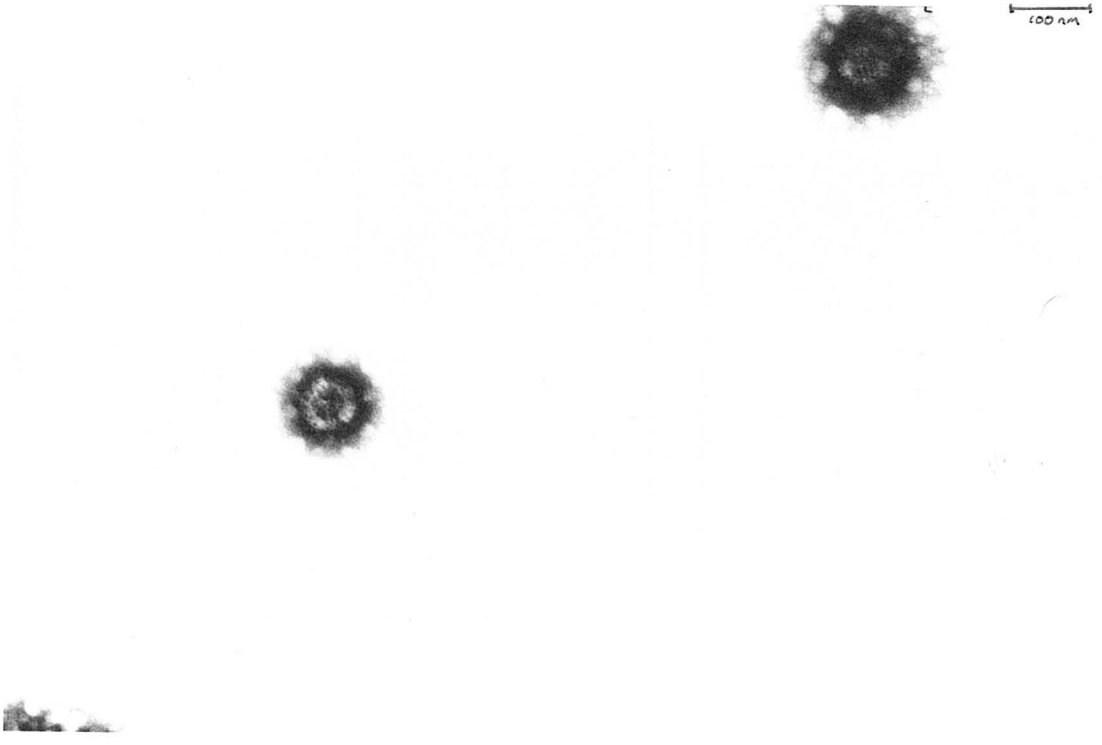

Fig. 4--Electron Micrograph of Dog Rotavirus.

as described in 2.4.1-a) was adsorbed to a concentration of 5×10^9 staphylococcal cells per ml. Such a concentration would absorb up to 5mg IgG per ml of cells. Goat anti-human rotavirus whole serum (supplied by B.A. Todd) was also adsorbed to staphylococcal protein A cells and so was normal rabbit IgG. These three preparations of IgG coated staphylococcal cells were washed twice and resuspended at an equal cell concentration in PBS of 5×10^9 cells per ml. A human rotavirus positive faecal specimen was suspended in PBS (20% suspension) and clarified by centrifugation at 5,000 rpm for 10 minutes. The supernatant was diluted by doubling dilutions in a titration series from 1/2 to 1/1024. These specimen dilutions were then reacted with the three preparations of IgG adsorbed staphylococcal cells and the degree of agglutination recorded (Table 2). The rabbit anti-SA_{11} rotavirus-staph. protein A preparation was 32 times more sensitive to human rotavirus antigen than was the goat anti-human rotavirus-staph. protein A preparation. The normal rabbit IgG adsorbed preparation gave no nonspecific binding of cells.

Upon repeated experiments the consistency and specificity of this test could not be relied upon. Large numbers of false positive results occurred. The development of RIA and ELISA systems for rotavirus detection, which proved to be more consistent and specific, replaced this technique. However, inclusion of more controls and inhibitors of nonspecific binding may render this a useful, rapid technique that could be easily used in field situations outside of a laboratory.

3.3 RIA

3.3.1 Preparation of Specific Antisera.

a) Rabbit anti-SA_{11} rotavirus. After immunization with 100µg of purified SA_{11} rotavirus and two boosts of approximately 100µg each, the rabbit was bled out. Antibody levels were screened by titrating dilutions of whole rabbit sera against a constant amount of SA_{11} rotavirus. The

TABLE 2

Staphylococcal Protein A - Rabbit anti-SA_{11} Rotavirus Agglutination

Dilution of Human Faeces Suspension	Adsorbed Antisera*		
	Rabbit Anti-SA_{11} Rotavirus	Goat Anti-Human Rotavirus	Normal Rabbit IgG
1/2	3+**	2+	-
1/4	3+	2+	-
1/8	3+	2+	-
1/16	3+	2+	-
1/32	3+	1+	-
1/64	3+	±	-
1/128	2+	-	-
1/256	2+	-	-
1/512	2+	-	-
1/1024	1+	-	-

* Antisera adsorbed to staphylococcal protein A.

** Grading of agglutination of Staphylococcus aureus: 3+ = high degree of agglutination; 1+ = low degree of agglutination; - = no agglutination.

rabbit sera was diluted in PBS from 1/10 to 1/20, 480 using doubling dilutions. Preimmunization sera was used in an equivalent dilution series as a control to check levels of nonspecific binding. Bound rabbit antibodies were detected by ^{125}I-labelled sheep anti-rabbit IgG. Results were recorded as the percentage of radioactivity bound after incubation and washing steps.

Over the period of immunization the level of specific antibody to SA_{11} rotavirus increased over preimmunization levels. Figure 5 shows the titration of the final bleed. The antibody level was high with an endpoint titre greater than 1/20,480. The graph of the titration shows a typical "mountain" effect caused by too much rabbit antibody being bound in the well causing steric interference of the sheep anti-rabbit binding. As the rabbit antisera is diluted out this effect is overcome and a true indication of the antibody levels is obtained (Maskill, PhD thesis).

b) Rabbit anti-Reovirus Type 3. The rabbit was immunized with 260µg of purified reovirus and 2 boosts of 100µg of reovirus each. Figure 6 shows the antibody levels as titrated against a constant amount of reovirus (0.4µg per well). Shown are the antibody levels of 12 days after the initial inoculation and prior to the first boost, and also antibody levels just prior to bleeding out the rabbit. It can be seen that specific rabbit anti-reovirus antibody levels increased greatly over the immunization period. The endpoint titre was in the order of $1/7 \times 10^5$. Again the "mountain" effect is evident.

c) Sheep anti-Rabbit IgG. A sheep was inoculated with 5mg of rabbit IgG and given 3 boosts of 500µg IgG each. Levels of specific sheep anti-rabbit IgG were determined by an inhibition assay. Doubling dilutions of sheep anti-rabbit sera (1/10 to 1/20,480) were incubated against a constant amount of rabbit IgG fixed to microtitre wells (0.5µg per well). After incubation of the sheep anti-rabbit sera dilution and appropriate washes, ^{125}I labelled, commercial sheep anti-rabbit IgG sera

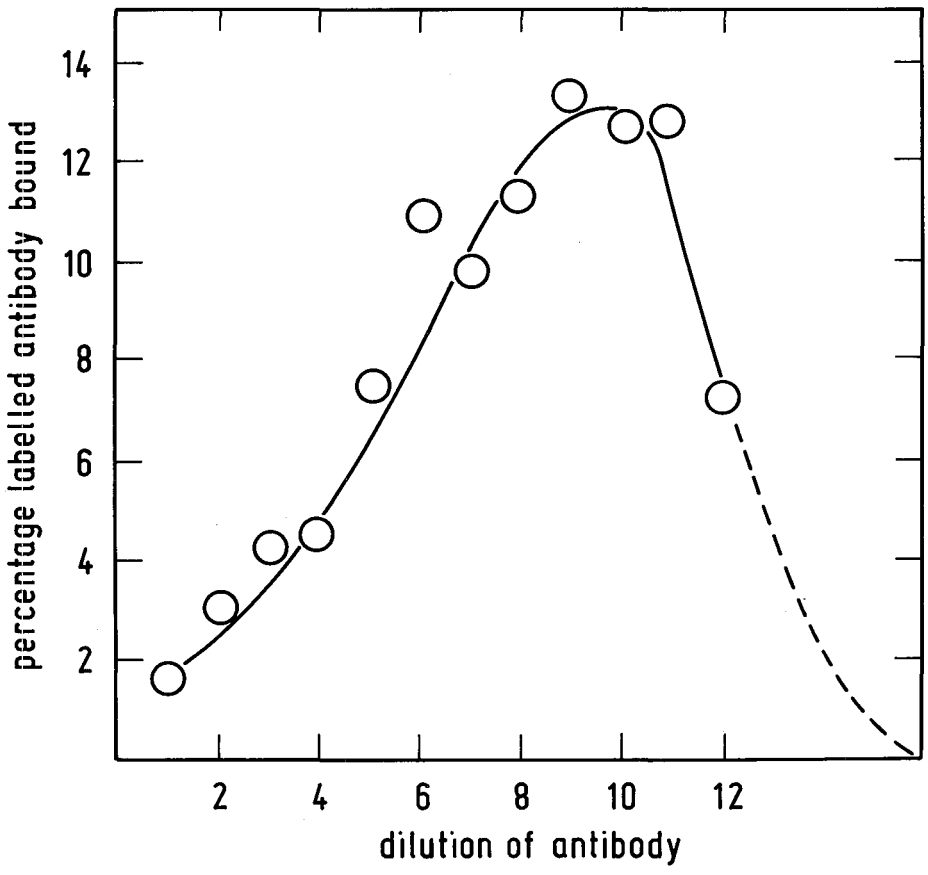

Fig. 5--Titration of Rabbit Anti-SA_{11} Rotavirus Antiserum. Doubling dilutions (starting at 1/10) of rabbit anti-SA_{11} were titrated against a fixed concentration of SA_{11} rotavirus. Bound rabbit antibodies were detected with ^{125}I-labelled sheep anti-rabbit and the bound radioactivity counted.

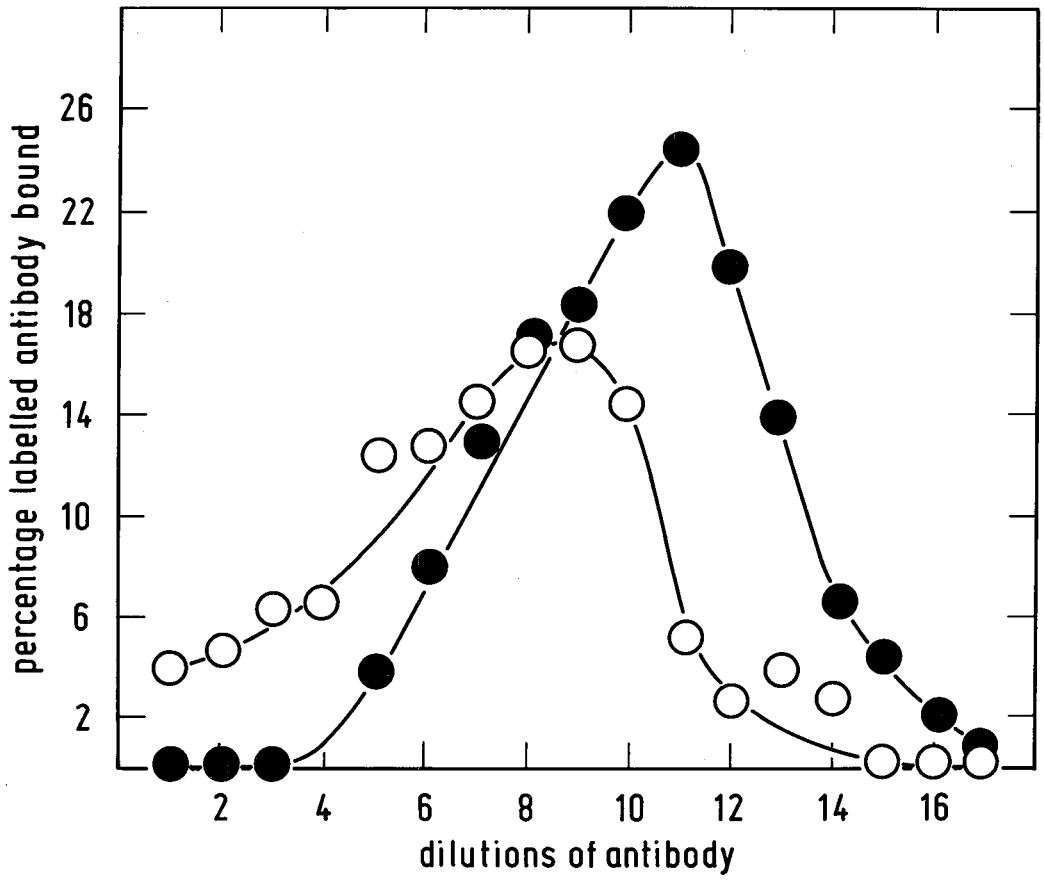

Fig. 6--Titration of Rabbit Anti-Reovirus Antiserum. Doubling dilutions of rabbit antiserum (starting at 1/10) were titrated against a fixed concentration of reovirus type 3 (0.4µg per well).

○ Titration of serum levels 12 days after initial inoculum.

◉ Titration of serum levels at end of immunization.

Bound rabbit antibodies were detected with ^{125}I-labelled sheep anti-rabbit and the bound radioactivity counted.

was incubated against the rabbit IgG at a constant dilution. Where there were specific sheep anti-rabbit antibodies (unlabelled) bound from the dilution series, inhibition of binding of the ^{125}I-labelled commercial preparation occurred and consequently the level of antibodies present in the immunized sheep sera could be determined. Figure 7 shows the level of ^{125}I-labelled sheep anti-rabbit IgG antibodies that bound when (1) pre-immunization sheep sera was used, and (2) when sheep sera at the end of the immunization procedure had been used. The immunized sheep sera inhibited binding of the labelled antibody at a 1/1280 dilution with a 50% inhibition at 1/160 dilution.

3.3.2 Labelling and Standardization of the Detector Antibody. A typical assay of ^{125}I labelled sheep anti-rabbit IgG titrated against rabbit IgG is shown in Figure 8. Dilutions of rabbit IgG were fixed to microtitre wells (25µl volumes) down to a final concentration of 39ng per ml. The labelled antibody, at a specific activity of 3.5×10^5 CPM per µg and a concentration of 4µg per ml was added to the wells (25µl). After incubation and washing steps, the percentage of radiolabelled sheep anti-rabbit that had bound to the well was determined. The preparation shown in Figure 8 had a rabbit IgG detection endpoint of less than 39ng per ml or 1ng per well. This was a typical preparation of ^{125}I-labelled sheep anti-rabbit IgG used as the detection antibody for RIA of rotaviral antigens.

3.3.3 Detection of Rotaviral Antigen. The method for rotaviral antigen detection in faecal specimens is outlined in section 2.4.4. Of the 67 specimens tested using the RIA system, 17 (25.4%) were positive for rotavirus antigen. These positive specimens came from four different host species: 8 human, 5 calf, 3 cat and 1 foal (Table 4). Of the rest of the specimens, 42 (62.7%) were negative as compared with known positive and negative control specimens, 2 (3.0%) were unclear being on the border line between positive and negative, and 6 (8.9%) which were assayed as rotavirus

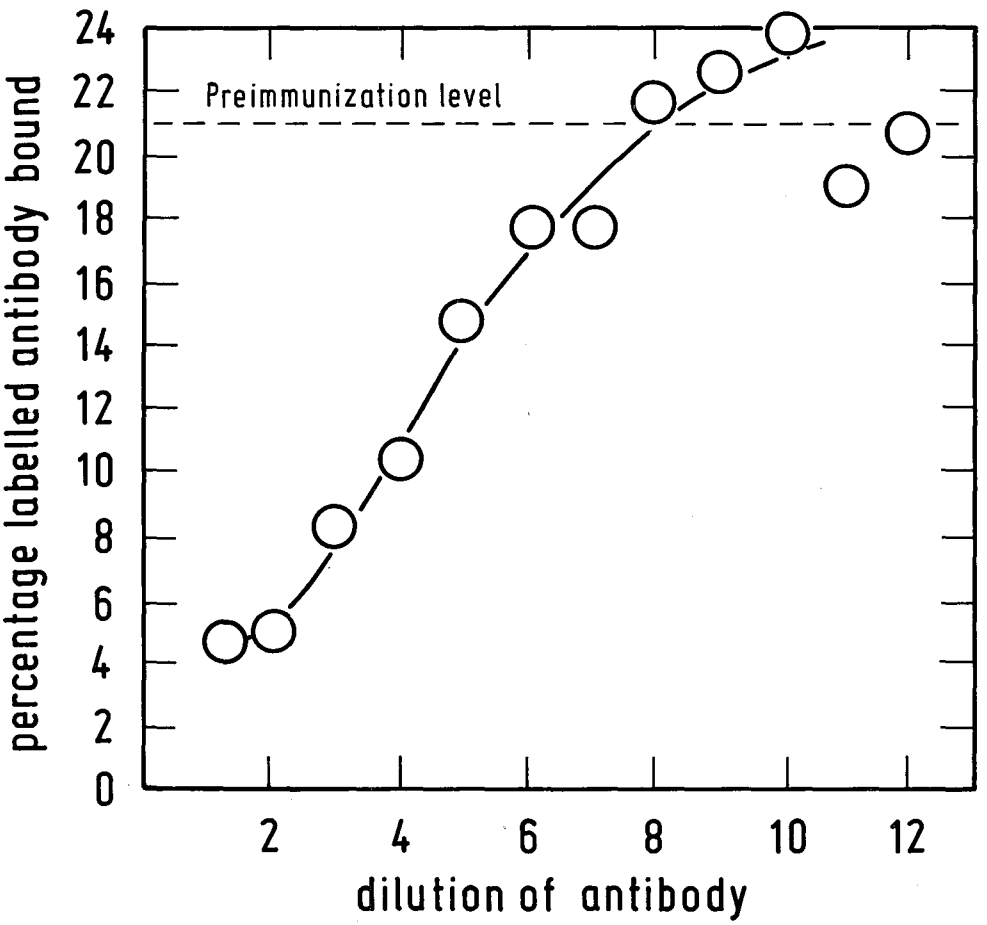

Fig. 7--Inhibition of ^{125}I-Sheep Anti-Rabbit (commercially prepared) with Doubling Dilutions of Unlabelled Sheep Anti-Rabbit (starting at 1/10) against a Fixed Concentration of Rabbit IgG (0.5µg per well). The pre-immunization level was determined using sheep antisera taken prior to immunization.

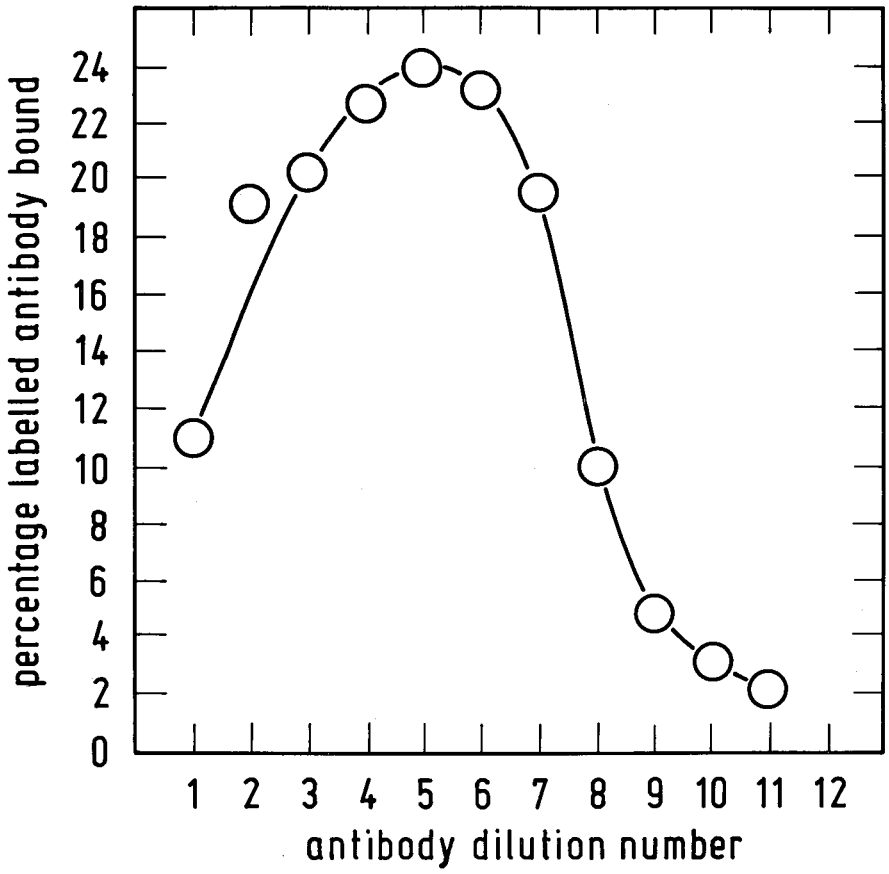

Fig. 8--Detection Limit of ^{125}I-Labelled Sheep Anti-Rabbit IgG. ^{125}I-labelled sheep anti-rabbit IgG titrated against doubling dilutions of rabbit Ig (1µg per well to 1ng per well). Labelled antibody was added at a concentration of 0.1 per well and a specific activity of 3.6×10^4 CPM.

TABLE 3

Faecal Rotavirus Screen by RIA

	Number	Percentage
Positive	17	25.4
Negative	42	62.7
Indecisive	2	3.0
False Positive *	6	8.9
Total	67	100

* Determined later by ELISA.

TABLE 4

Rotavirus Host Species as Detected by RIA

	Number Positive	Total Number of Specimens	Percentage Positive
Human	8	18	44.4
Bovine	5	34	14.8
Feline	3	10	30
Equine	1	5	20

positive were in fact false positives when screened by ELISA (Table 3). Figure 9 shows the range of results obtained for a number of specimens for 3 different assays (a, b and c). A number of results fell in the border area between being clearly rotavirus positive or negative.

3.4 ELISA

3.4.1 Conjugation and Standardization of Immunoglobulin and HRP.

Horse-radish peroxidase was successfully conjugated to a number of different antibody types (Table 5). Generally the method of Nakane and Kawaoi (1974) was successful and was most often used in conjugations. The modification of this method by Nakane (1978) where a low pH is used instead of FDNB to block enzyme self-linkage, also proved to be successful. The efficiency of conjugation was determined from the R_z values taken for each fraction as it passed through the sephadex column, the peak R_z fractions were pooled and used in ELISA experiments. Table 5 gives the R_z values of conjugate preparations made and their equivalent enzyme to antibody molar ratio as determined by the method in section 2.5.2. The average ratio of enzyme to antibody was 0.66 enzyme molecules to every antibody molecule.

When the HRP was measured by spectrophotometric means at 403nm, as it passed through the sephadex column, it was seen to peak in the same fractions as the peak for IgG (read at 280nm), indicating that the majority of the HRP was being conjugated to the IgG and was not remaining as free enzyme (Fig. 10). Free HRP enzyme came out of the column some five fractions after the peak conjugate fraction.

Determination of the dilution at which the conjugate preparation could then be used was made by checkerboard titration of conjugate dilutions assayed against dilutions of antigen (Fig. 11).

The time course of the HRP-substrate colour reaction was followed in order to determine the optimum time for reading an ELISA result. From

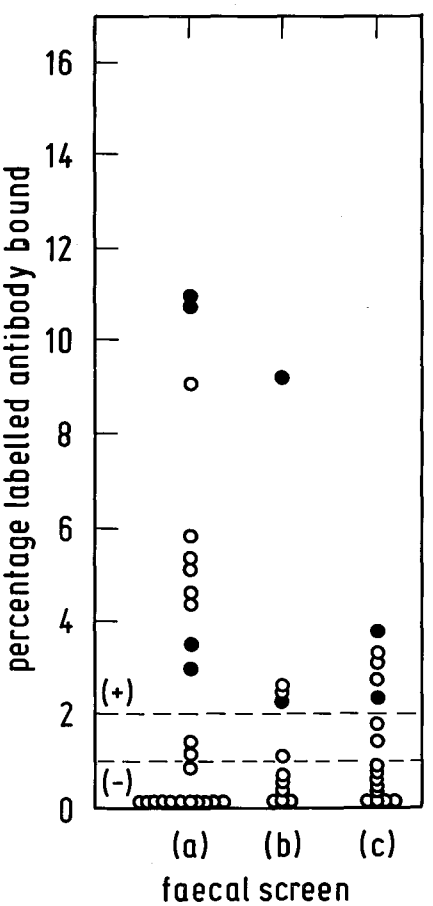

Fig. 9--Detection of Rotaviral Antigen. Faecal specimens from humans and animals were reacted with rabbit anti-SA_{11} antibody and then ^{125}I-labelled sheep anti-rabbit IgG. The results of 3 assays (a, b and c) are shown.

O Test specimens.

● Known rotavirus positive specimens.

Positive results were recorded as showing greater than 2% binding of added radiolabel ^{125}I.

TABLE 5

Antibody-Enzyme Conjugates

Antibody	Conjugation Method	$R_z \frac{403}{280}$	Enzyme/Antibody
Rabbit x Dog IgG	FDNB	0.11/0.41	0.4
Rabbit x Cat IgG	FDNB	0.84/1.08	1.4
Sheep x Mouse IgG (1)	FDNB	0.37/3.72	0.13
Sheep x Mouse IgG (2)	FDNB	0.1 /0.4	0.38
Sheep x Rabbit IgG (1)	FDNB	0.25/0.59	0.68
Sheep x Rabbit IgG (2)	FDNB	1.43/3.92	0.51
Sheep x Rabbit IgG (3)	FDNB	3.77/8.78	0.62
Sheep x Rabbit IgG (4)	FDNB	2.01/5.11	0.56
Sheep x Rabbit IgG (5)	pH	0.25/3.4	0.12
Sheep x Rabbit IgG (6)	pH	0.99/6.09	0.21
Sheep x Human IgG (1)	FDNB	2.55/7.2	0.50
Sheep x Human IgG (2)	FDNB	1.49/1.67	1.63
Sheep x Human IgA	FDNB	1.52/1.76	1.49

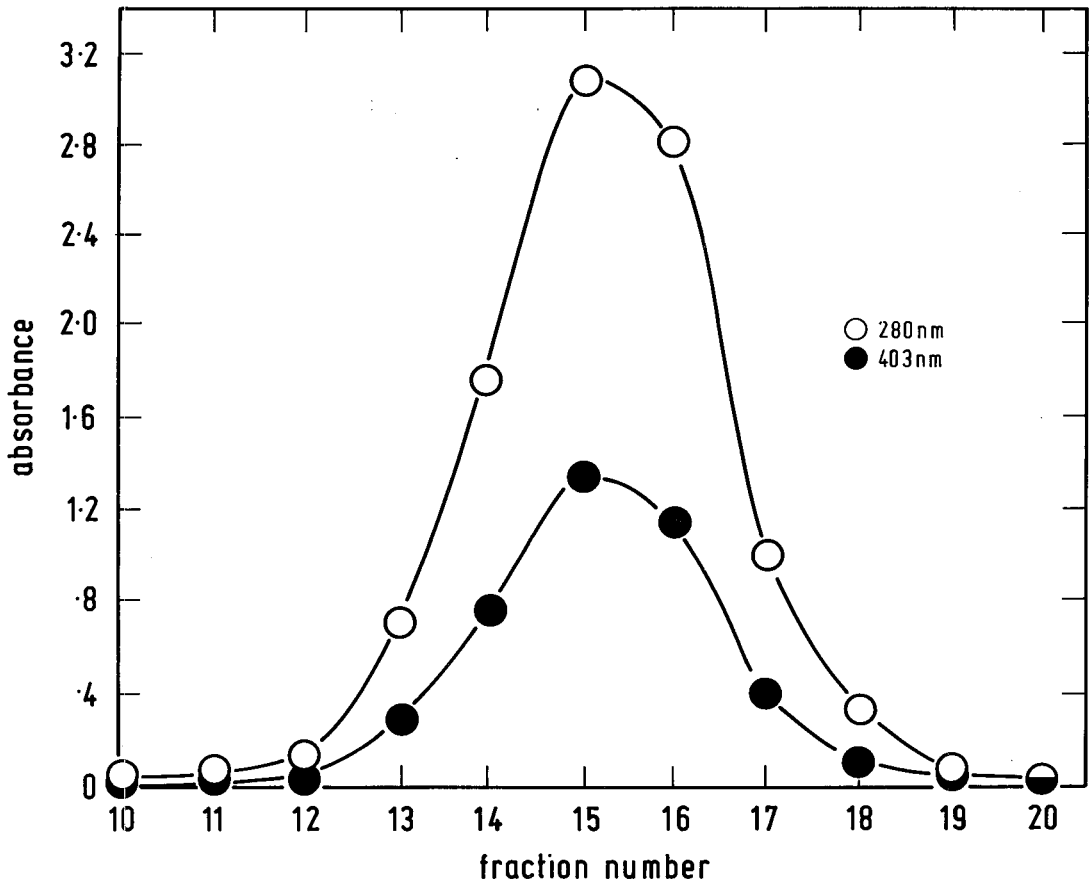

Fig. 10--Separation of Sheep Anti-Rabbit IgG-HRP from Free HRP.

○ Conjugated antibody-HRP enzyme purified on a sephadex G-100 column collected in 1ml fractions.

● 403nm spectrophotometric readings detected the heme portion of HRP enzyme.

Free HRP enzyme was found in fractions 25-27.

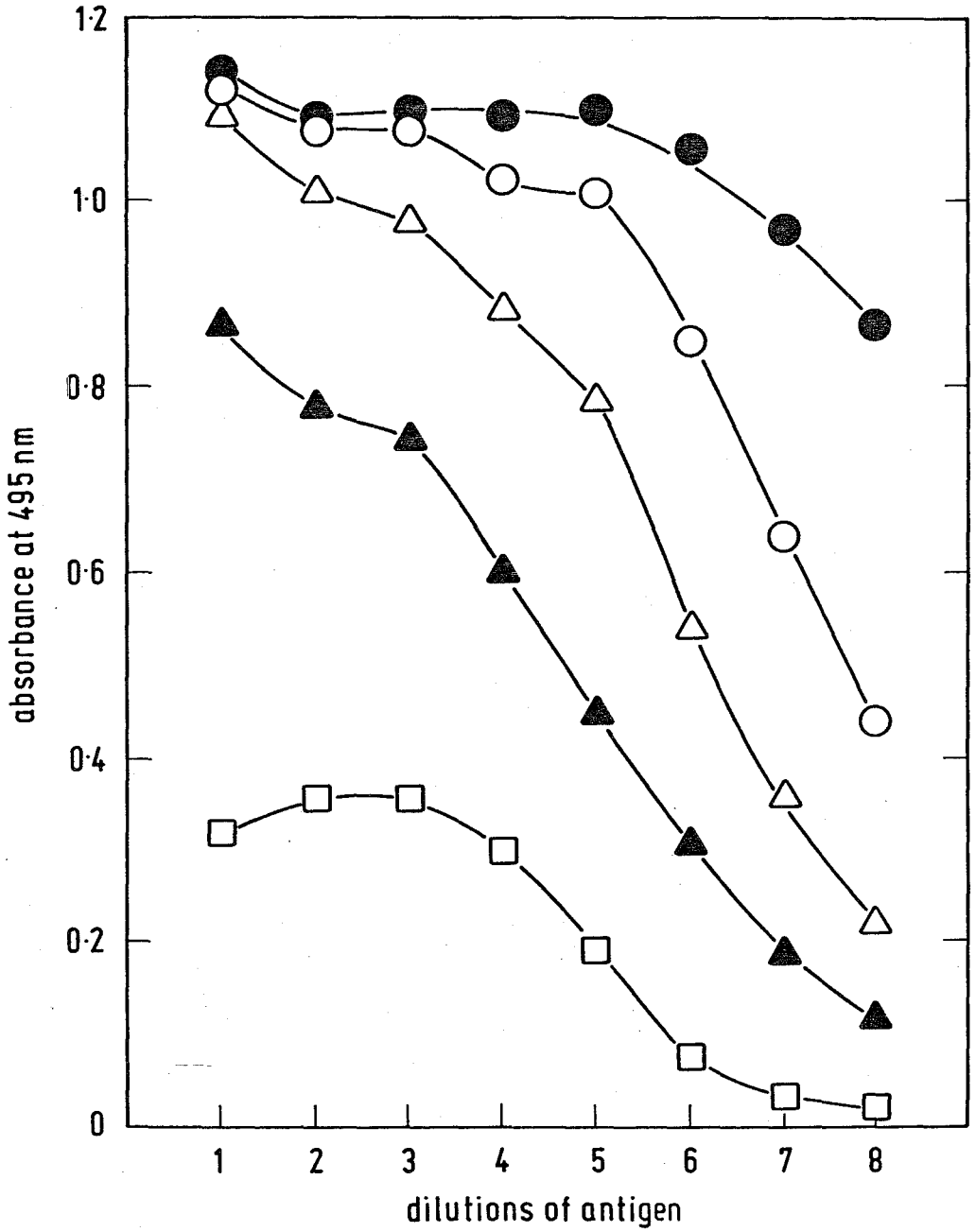

Fig. 11--Dilution of HRP Conjugated Antibody versus Antigen Dilutions.

Antigen dilutions - rabbit IgG (1.25µg to 10ng per well).

Antibody dilutions - sheep anti-rabbit IgG-HRP.

● 1/20 ○ 1/80

△ 1/320 ▲ 1/1280

□ 1/10,240

Sheep anti-rabbit IgG-HRP was used in antigen detection assays at a final dilution of 1/500.

Figure 12 it can be seen that after an initial drop in absorbance, the development of colour from the OPD chromagen reaction causes a rapid increase in absorbance for the first eight minutes of the reaction and then continues to increase slowly for a further twelve minutes, until after twenty minutes the reaction has reached a plateau and there is no further increase in absorbance. The limiting factor in this reaction is the chromagen reaction resulting in the observed colour. There is a limit to the degree of colour that can be produced as is shown by the leveling off of the absorbance readings. In an antigen detection assay, the strongly positive specimen may reach this upper limit of colour development quite quickly, but if left to incubate for a longer period of time the weakly positive specimen would continue to develop colour while the strongly positive specimen remained constant. This would effectively mask the difference between the two specimens if left long enough. Also nonspecific binding of a low level can give a relatively strong colour reaction if left long enough. It was decided that an incubation time for substrate reaction of approximately 15 minutes would allow for strong colour development of positive specimens, would optimise the difference between strongly positive and weakly positive specimens and would not be long enough for significant colour development from small amounts of nonspecific binding.

3.4.2 Control of Nonspecific Binding in the Antigen Detection System. One of the main problems encountered when screening faecal specimens for rotavirus antigen is nonspecific binding of the intermediary and detector antibodies to either the faecal material or to the plastic of the microtitre wells. In RIA such nonspecific binding was dealt with by either blocking sites on the plastic with gelatin or BSA or else by incorporating foetal calf serum (FCS) into the diluent of the antisera. By incubating preimmune sera and detector antibody conjugate directly with faecal specimens, as controls, such nonspecific binding could be detected.

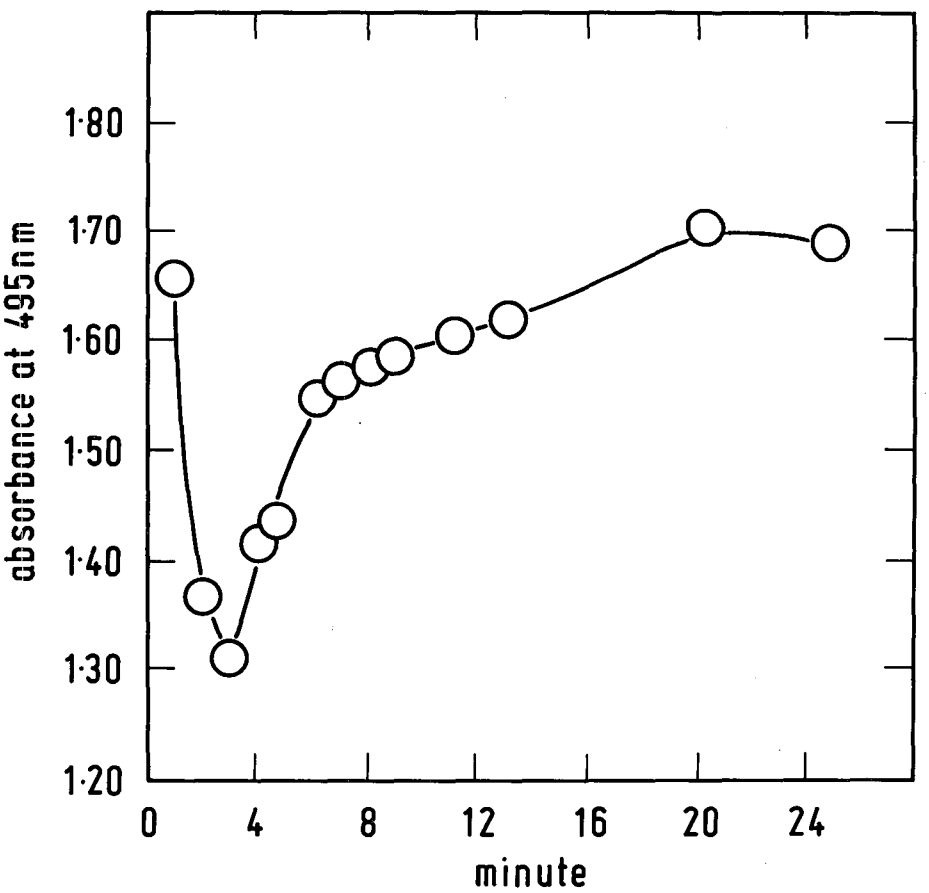

Fig. 12--Time Course of Colour Development from HRP-Substrate Reaction. Rabbit anti-dog IgG-HRP (1/100 dilution) was added to OPD substrate (0.4mg per ml of buffer). A reaction time of 15 minutes was allowed in antigen and antibody detection assays before results were read.

TABLE 6

Effect of FCS in Diluent for Intermediary and Detector Antibodies on Nonspecific Binding (NSB)

FCS in Rabbit Anti-SA_{11}	FCS in sheep anti-rabbit-HRP			
	1%	10%	20%	50%
1%	High (NSB)	low	low	low
10%	High	low	low	low
20%	Moderate	low	low	low
50%	Moderate	low	low	low

TABLE 7

Effect of FCS, High Salt and Detergent on Nonspecific Binding of the Detector Antibody

Dilution of Antigen (doubling dilutions)	Sheep anti-rabbit-HRP Serum with 10% FCS*		Sheep anti-rabbit-HRP Serum without FCS*	
	Uninfected Cells	SA_{11} Infected Cells	Uninfected Cells	SA_{11} Infected Cells
1	1+	3+	4+	4+
2	1+	3+	4+	4+
3	1+	3+	4+	4+
4	1+	3+	4+	4+
5	1+	2+	4+	4+
6	1+	1+	4+	4+
7	-	1+	2+	4+
8	-	1+	1+	2+
9	-	1+	1+	2+
10	-	1+	1+	1+
11	-	1+	-	1+
12	-	-	-	1+

- = no colour development.

1+ = low colour development.

4+ = high colour development.

* High salt concentrations (0.5M NaCl) and detergent (0.05% Tween 20) were included at all stages.

3.4.4 Detection of Rotavirus Antigen. The ELISA system used for the detection of rotavirus antigen whether from tissue culture, faecal specimens, or purified virus, was that described in methods section 2.5.3. The system was first checked against a number of known positive and negative specimens and found to give a 100% correlation with expected results. The sensitivity of the assay was determined by assaying doubling dilutions of purified SA_{11} rotavirus. The result of this assay was a detection limit of 15ng in a 25µl volume or 0.6µg per ml.

The detection of purified viral antigen, however, may not be a true indication of what happens in the assay of untreated faecal suspensions. Consequently a number of known positive specimens were diluted out and the endpoint titre determined for each. The results ranged from very weakly positive specimens, with an endpoint titre of 1/40, through to strongly positive, with endpoints of 1/1280. The majority of specimens that were titrated gave endpoints between 1/80 and 1/160.

Faecal specimens for antigen detection were collected as described in section 2.1. Overall 70 human specimens, 104 cat, 75 dog, 70 calf, 15 foal and 5 piglet specimens were screened for rotavirus antigen using the ELISA system. Of this total of 339 specimens, 115 were found to contain rotavirus antigen, or 33.9%. The breakdown of the different host species can be seen in Table 8.

Of the total of 339 specimens screened for rotavirus antigen by ELISA, 185 were from hosts that showed some symptoms of a gastroenteritis-type infection. Ninety-eight of these were rotavirus positive (53%). A further 17 specimens were rotavirus positive from hosts that showed no symptoms of a gastroenteritis type infection (Table 9).

The detection of rotaviral antigen in the various host species differed markedly, ranging from 82.9% in humans to 13.3% in dogs. Of the hosts that showed gastroenteritis-like symptoms and were in fact rotavirus

TABLE 8

Detection of Rotavirus Antigen in Faecal Specimens

Host Species	Number Positive	Total Number	Percentage Positive
Human	58	70	82.9
Feline	29	104	27.9
Canine	10	75	13.3
Bovine	10	70	14.3
Equine	7	15	46.7
Porcine	1	5	20
Total	115	339	33.9

TABLE 9

Relationship of Symptoms to Presence of Rotavirus Antigen

	Rotavirus Positive		Rotavirus Negative	
Host Species	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
Human	56	2	10	2
Feline	19	10	15	60
Canine	8	2	29	36
Bovine	7	3	22	38
Equine	7	0	8	0
Porcine	1	0	3	1
Total	98	17	87	137

positive, there were 84.8% for humans, 55.9%, cats; 21.6%, dogs; 24%, calves; 46.7%, foals and 25%, piglets.

There were a number of occasions when a child was admitted to hospital for infantile gastroenteritis, was found to be positive for rotavirus and had been exposed to a pet animal that had been previously ill. Specimens from these animals were always rotavirus negative. Likewise, there were two occasions when human specimens were obtained from owners of animals that had been shown to have a rotavirus infection but these specimens were also negative.

One infant repeatedly gave rotavirus positive faecal specimens over a period of 5 months. Initially specimens were collected daily from the time the baby was 3 months old. After two months specimens were collected every two weeks. Rotavirus particles were never seen by EM and no dsRNA could be detected by PAGE. The ELISA system was modified to include a capture antibody (goat anti-human rotavirus) which was dried onto a microtitre well and then a faecal suspension was added to react with the specific capture antibody; the rest of the assay proceeded as normal. Some specimens were reduced in their colour development but were still clearly positive for rotavirus. No symptoms ever developed although a number of stools were relatively loose. Other members of the family were also tested for rotavirus infection with the result that the two school age children were both positive, as was the father but the mother was negative. A litter of golden labrador pups (one-month old) were also assayed for rotavirus. Initially these specimens were all negative but one month after the first positive infant specimen, the pups also became rotavirus positive. Unfortunately these pups were given away and contact was lost. No rotavirus particles were observed by EM and dsRNA was not assayed for. The final proof of rotavirus infection in this intra-family "epidemic" was not made and so these results have been kept separate from the rest of the survey.

3.4.5 Detection of Rotavirus Antibody. Serum taken from third-year Microbiology undergraduate students was diluted 1/20 in PBS and incubated against SA_{11} rotavirus infected cells fixed to microtitre wells. Of the 31 sera tested, 12 (38.7%) showed evidence of antibody titres greater than 1/20 for rotavirus. Upon titration of these 12 sera against a constant dilution of SA_{11} antigen, one had a titre greater than 1/2560, three were greater than 1/640, three had titres of 1/320, three had 1/160, one had 1/80 and one had an endpoint titre of 1/40.

3.4.6 Overall Detection of Rotavirus from Human and Animal Survey. Over the three year period of collecting and screening of faecal specimens for rotavirus, in human and five animal species, a number of techniques were used. The three principle techniques were EM, RIA and ELISA. By the completion of the study a total of 427 specimens had been collected and assayed. Of these, 148 were positive for rotavirus. Table 10 shows the incidence of rotavirus in the various host species surveyed and the relationsip of symptoms to detection of virus. Of the total of 427 specimens, 225 came from a host with symptoms of a gastrointestinal infection, and 119 of these were actually diagnosed as having a rotavirus infection. Of the rotavirus positive specimens, 29 were from a host that showed no clinical evidence of an infection

TABLE 10

Overall Rotavirus Detection in Various Host Species

Host Species	Total Number of Specimens	Number Rotavirus Positive (percentage)		Percentage of Symptomatic Specimens Positive
Human	80	67	(83.8)	85.5
Feline	115	32	(27.8)	54
Canine	86	32	(18.6)	21
Bovine	101	14	(13.9)	27.3
Equine	35	16	(45.7)	47.1
Porcine	10	3	(30.0)	-
Total	427	148	(34.7)	52.9

4. DISCUSSION

Part II of this thesis deals with the techniques used in the detection and surveillance of rotavirus in human and animal populations. Although most of these techniques have been well characterized and standardized in the literature, some modifications and refinements of the techniques had to be made in order to apply them to our particular situation. The ELISA system, in particular, tended to evolve during the survey period as problems of nonspecific binding were met and overcome and the improvements in conjugation procedures were made. The methods presented were the final methods used in the study. The introduction of FCS, detergent and high molar salt in diluents and washing steps improved the accuracy of the system. Also the use of preimmunization sera and reacting conjugated antibody directly with faecal material as controls readily identified any problems of nonspecific binding. The possibility that peroxidase enzyme was present in some specimens and thereby causing nonspecific reaction with the substrate was also considered but when specimens were reacted directly with substrate, no reactions occurred. The realization that some of the RIA positive results were in fact false positives came with the introduction of control steps when specimens were rescreened using the ELISA system. This illustrates the value of having adequate control steps in an assay to detect any problems or deficiencies. It also helped to determine the point in the assay at which the problem has been occurring and a consequent plan of action.

All techniques used proved to be useful in rotavirus diagnosis. The ELISA system, because of its simplicity, sensitivity, accuracy and rapidity became the favoured technique and was used as the main diagnostic technique after the first year of the study.

A large number of specimens that were positive by ELISA were negative when screened by EM. Likely explanations for this are: 1) the insensitivity of EM detecting only to a lower limit of approximately 10^6 particles per ml; and 2) that ELISA will detect virus antigen as well as whole virus particles, whereas EM will only detect whole virus. It would be expected that at some stages during the infection period that virus antigens may be present in faecal specimens without the shedding of whole virus particles. In tissue culture propagation of rotavirus one of the main problems encountered is the partial replication of virus without the production of complete and fully infectious particles. The same situation may apply in the natural host at certain stages of the infection.

Detection of rotavirus antigen using staphylococcal protein A bound with specific anti-rotaviral antibody as an agglutination test was attractive because of its simplicity and speed. Problems of nonspecific aggregation were never overcome and consequently it was not used routinely. However, some of the modifications introduced into the ELISA system to block nonspecific binding (i.e., FCS, detergent and high molar salt) may also be applicable to the staphylococcal protein A agglutination system. Such modifications were not attempted but would be worthy of further examination. If the staphylococcal-agglutination system could be modified to give consistent, and reliable results it would be a very useful diagnostic technique for field situations. The only equipment and reagents needed are glass slides, some staphylococcus with specific antibody bound and perhaps a magnifying lens to help observation of agglutination. It is certainly very rapid taking only 10-30 minutes to perform, needs no elaborate equipment and is very simple to perform and interpret.

As discussed in the introduction (1.4.5) the accuracy and sensitivity of the assay system when using immunological reagents is only as good as the reagents used. The antisera prepared in rabbits and sheep for both

the RIA and ELISA systems was of high titre and specificity for the antigen desired. The value of having an antisera with a very high titre of specific antibody is that it can be used at a dilution such that other antibodies that may be present in the antisera will be diluted out, consequently reducing the level of nonspecific antibodies. In this respect the two antibodies used throughout the survey, rabbit anti-SA_{11} rotavirus and sheep anti-rabbit IgG, were both of relatively high titres. The rabbit anti-SA_{11} rotavirus was used throughout at a 1/1600 dilution. This could in fact have been diluted further as seen from the titration curve (Fig. 5). A 1/1600 dilution is on the side of the curve that indicates a very high level of antibody still present. This may have in fact been a cause of some of the nonspecific binding that occurred. At this dilution, with such a high level of antibody, there would tend to be antibody molecules present that were not as highly specific in their affinity for rotavirus antigen. By diluting the antiserum further, the antibody with high specificity would predominate and would yield an assay system of much higher specificity (Thongkrajai, thesis, 1980). However, to balance this, there may be value in having some less specific antibody present to detect rotavirus of possibly different serotypes. A highly specific assay would only detect a very small range of antigens and so may be restricted to detecting only one serotype. In our survey we were interested in determining the incidence of rotavirus in the human and animal populations and not necessarily specific serotypes. It would have been better to use the rabbit anti-SA_{11} rotavirus antiserum at 1/3200 - 1/6400 dilution to reduce the nonspecific antibody concentration and yet not make it so specific that only outer capsid serotyping antigens were being detected. The sheep anti-rabbit IgG-HRP was used at a 1/500 dilution. There was very little problem of nonspecific binding with the conjugated antibody as long as FCS, detergent and high salt concentrations were used in the diluent.

Although no direct comparison of sensitivity between EM, RIA and ELISA was made it was evident that ELISA was the most suitable assay for our requirements of a simple, rapid, sensitive and accurate assay system to detect rotavirus in faecal specimens.

In two comparative studies of EM and ELISA by Brandt, et al., (1981) and Grauballe, et al., (1981), ELISA was found to be the more sensitive technique and was favoured because of its rapid screening of large numbers of faecal specimens in a single test. It was easy to perform, showed good reproducibility and needed no elaborate equipment with the end result being adequately read by eye. Brandt, et al., (1981) had problems with nonspecific binding resulting in 73% of all positive results proving to be false. They overcame this problem by including a preimmunization sera as a control and using a capture antibody rather than fixing faecal material to the microtitre wells. One of the possible explanations for nonspecific binding propounded by Brandt, et al., (1981) was that bacteria in the faecal material may bind antibody in an analogous way to Staphylococcus aureus protein A. Yolken and Stopu (1979) found that pre-treatment of faecal specimens with the mild reducing agent N-acetylcysteine markedly reduced nonspecific binding but did not reduce the specific ELISA activity due to rotavirus. Brandt, et al., (1981) found that such pre-treatment was not needed for their study as the steps taken eliminated false positive reactions. For this present study the use of a mild reducing agent for pre-treatment of specimens was not tried but may have been a useful method for reducing nonspecific reactions. The inclusion of a capture antibody was tried but did not reduce nonspecific reactions and appeared to reduce the sensitivity of the assay. After standardization of the ELISA system, determining incubation times and incorporating adequate control steps, we were satisfied that the ELISA test was giving accurate, reliable and sensitive results for screening faecal material for rotavirus antigen and human sera for specific anti-rotavirus antibody.

Because the RIA and ELISA for detecting rotavirus antigen were using the same preparations of immunological reagents, the RIA, with the modifications introduced to the ELISA, would most likely have proved to be just as reliable. The false positives given by RIA would have been detected if adequate controls had been incorporated. The system was not persisted with because of its requirement for radioactive label which restricted its usefulness to laboratories that are well equipped for handling radioactive material. However, for a more quantitative type of study, the RIA system would be more useful than ELISA. For instance, if a study on serotyping and the relative affinities of particular antibody to antigen of the different types was being studied the answers given by RIA of actual degrees of binding and the affinity of the particular antibody to antigen that can be calculated would be much more useful. ELISA is a system that can only be semi-quantitative as it is used at present. The only way it could be made fully quantitative in an equivalent way to RIA is for the specific amount of antibody added to a specimen to be known, for the precise amount of enzyme conjugated to that antibody to be known and for the rate of reaction of enzyme and substrate to be measured, rather than a simple absorbance reading to be taken as at present. Such measurements would mean a precise amount of antibody bound to the antigen could be calculated and from a predetermined standard curve, an equivalent amount of antigen be known. Such measurements and calculations may even allow for antibody affinities to be calculated as for the RIA system. However, such measurements again require more elaborate equipment than is found in the average diagnostic laboratory and so such work would be restricted to larger reference laboratories or research institutions.

The method of Nakane and Kawaoi (1974) for conjugation of HRP enzyme to IgG molecules generally worked very well. Figure 10 shows that most, if not all the HRP, was conjugated to IgG. For this particular conjugation

there was one enzyme molecule to approximately every second antibody molecule. This indicates that the efficiency of conjugation could be bettered by increasing the amount of HRP enzyme added to the reaction mixture, or by decreasing the excess amount of IgG present. This would create conditions that would increase the probability of every antibody molecule being conjugated with an enzyme molecule. As it was, in only 3 of the conjugations listed in Table 5 was there a 1:1 ratio of enzyme to antibody. Unlabelled antibody decreases the sensitivity of the assay as it blocks antigen to labelled antibody.

The pH method was tried as an alternative to the use of FDNB which was found to cause a type IV hypersensitivity reaction upon contact in a sensitized individual. The pH method worked successfully but not as well as the FDNB method. However, only 2 conjugations by this method were tried and a greater number may well reveal a greater efficiency. Other members of the Department of Microbiology that have tried the pH method found it to be as good as with FDNB.

The survey of faecal specimens for rotavirus particles and rotavirus antigen, showed rotavirus to be a common infectious agent of all the host species screened. The host to show the highest degree of virus detection was the human infant. An overall virus detection of 83.8% positive for rotavirus is not surprising when it is realized that most of these specimens came from children hospitalized for infantile gastroenteritis. The majority of these specimens were taken over the winter period when infantile gastroenteritis reaches almost epidemic proportionsthroughout New Zealand. Of all the specimens that were received from symptomatic children, 85.5% of these, in fact, proved to be positive for rotavirus.

The animal specimens tended to come largely from symptomless hosts or situations where there had been infrequent cases of diarrhoea. For instance with the calf specimens there were a number of occasions where 1 or

2 calves in a herd of 10-12 would be "scouring" and the rest appeared normal. Only in 2 situations did rotavirus appear to cause an epidemic disease type situation as is common in man. In one litter of greyhound pups, all nine pups developed symptoms of severe diarrhoea, vomitting and fever. From 10 faecal specimens received, 7 were positive for rotavirus and 3 were negative. The animals were kept together in a close environment and so it was not possible to determine from which individual animal, specimens had come. Although only 7 of the 10 specimens were rotavirus positive, it is probably safe to assume that all 9 animals were infected by rotavirus. The second situation was with a herd of foals that were being bred and raised on one property. There was a continual problem over the summer foaling period of 1-week old foals developing severe diarrhoea. As in the case with the greyhound pups, not all the specimens were found to be rotavirus positive. These two situations appear to indicate that during the period a host is infected with rotavirus there may be short intervals when rotavirus cannot be detected in faecal specimens either because virus is not being excreted or it is not in sufficient quantity to be detected.

The bovine, feline and canine specimens, however, were collected largely from symptom-free animals. In symptomless cats, out of 70 specimens tested only 10 were positive (14.3%), in dogs only 5.3% and in calves 7.3% of symptomless animals were rotavirus positive. It can be concluded that rotavirus can be carried in young animals without any observable symptoms but that this tends to occur at a fairly low rate. When these figures are compared with the percentage of symptomatic specimens it becomes obvious that rotavirus is normally associated with some observable symptoms. Amongst the animal populations alone the overall incidence of rotavirus infection in both symptomatic and asymptomatic hosts was 27.2%. The low incidence of rotavirus in symptomless animals may in fact reflect the limits of the detection systems rather than low levels of asymptomatic

infection. When it is considered that symptomatic animals in close contact with known rotavirus positive animals, may be negative for rotavirus, it is not surprising that rotavirus is not commonly detected in symptomless animals.

In animals the disease was usually evidenced by similar symptoms to those found in humans. Diarrhoea was the most common symptom, particularly in calves where the faeces became very fluid, at times mucoid, tended to be light brown or yellow in colour and had a "sweet" smell. The litter of greyhound pups were most severely stricken of any of the animal hosts observed. The pups were one week post-weaning when convulsions and diarrhoea developed, they soon became feverish and very lethargic. These symptoms persisted for five days after which all the pups fully recovered and a year later were fully healthy and well developed. Foals infected with rotavirus were symptomatically very similar to calves. The scouring developed within the first two weeks of life and persisted for 2-5 days. On one particular property the owner believed the virus was being brought onto the property by mares from other areas and that foals never fully developed subsequent to a scouring outbreak. On this property, the owner always kept the foals on one paddock year after year. It was suggested to the owner that because rotavirus is a fairly stable virus it may be able to persist in the paddock over the year and infect new susceptible foals in the following breeding season. In following years newborn foals were kept in different paddocks and scouring was noticeably reduced with less than 6 specimens being received over the two-year period. Other factors may have been involved in this reduction such as fewer infected mares being imported, or increased awareness leading to better handling and housing of animals. Build up virus in a paddock cannot be proven from these observations but it is supported by dairy farms that either had a history of scouring calves or tended to be relatively free year after year.

Cats were never observed to have a "full-scale" rotavirus infection but always appeared to be asymptomatic. Rotavirus from this study could not be said to be a severe problem in kittens but the relatively high detection rate (27.8%) could be significant if humans were able to contract a rotaviral infection from young animals.

It has been shown in this study that rotavirus infection is relatively common in two animal populations that have a high degree of contact with young children -- namely kittens and pups. It is not known conclusively whether animal rotavirus is able to infect young children but there is a large amount of circumstantial evidence, particularly with cross transmission studies, that indicate such a possibility. Therefore a better understanding of incidence of rotavirus and its relationship to concurrent human rotavirus should be made for these two animal populations. It would also be valuable to correlate any changes in the rotavirus type that is dominant in the human population with types that are isolated from animals in previous and subsequent years to see if there is any natural cross transmission of important types.

To date there have been no reports in the literature of a persistent rotaviral infection of either human or animal hosts. It was therefore a surprise to find one infant that was persistently rotavirus positive by ELISA for a period of 5 months. Rotavirus was never observed by EM and no dsRNA could be detected by PAGE. Two possibilities could explain this finding. Firstly, there was in fact a persistent infection that never developed into a clinical disease. Virus was not excreted as whole particles but only viral proteins and antigens, this would explain why no virus was seen by EM and no dsRNA was detected. The second possibility is that the ELISA was giving a false positive reaction and there was no rotavirus infection. Evidence is strongly in support of the first possibility. When a capture antibody was introduced into the ELISA system so that no faecal

material other than rotaviral antigen would remain the results did not significantly alter. Preimmunization sera used as a control did not bind non-specifically to the faecal material. Specimens of sera from the infant would have been helpful in giving some idea of the immunological status of the individual but were not obtained. It is believed that this child had a persistent infection of rotavirus but final proof was not made.

The antibody studies of the young adults, although few in sample number, did indicate that some adults have antibodies to rotavirus at relatively high levels. Four of the individuals had antibody titres greater than 1/640. In a study by Elias (1977) it was found that average antibody titres reached a peak of 1/160 in the 1-3 years age group as determined by fluorescent antibody. These antibody levels slowly dropped till they were undetectable in age groups greater than 70 years. In the 20-29 years age group average antibody titres were between 1/20 and 1/40. In serological studies previously done on two Otago populations -- Port Chalmers and Milton, it was shown that there was a relatively high level of humoral antibody against rotavirus in all ages but especially in the 20-30 years age group. Both studies show an increasing level of humoral antibody throughout childhood with a sharp rise in the age group containing the majority of parents with young children. It was suggested that this reflects exposure to their infected infants (Holdaway, et al., 1982). In the present study of 31 sera tested, 10 had antibody titres of 1/160 or greater. The ages of these students ranged from 20 to 23 years. As far as is known, none of the students had children of their own or were in close contact with infants. This then would imply that rotavirus infection may be common among young adults regardless of their parental status. Elias (1977) concluded that because of the falling pattern of rotavirus titre with age, that rotavirus infection was not persistent and reinfection does not occur, or that if rotavirus infection is repeated, it does not

provide much antigenic stimulus. The results of our present survey indicate that either there are individuals with persistent rotaviral infections giving a constant immunological boost or that individuals are being reinfected with either the same virus or one of a different serotype. Both situations may in fact occur. A possible persistent infection has already been reported in this section and there are a number of reports of recurrent rotavirus infections (Rodriguez, et al., 1978; Fonteyne, et al., 1978). There have been a number of human serotypes reported (Hodes, 1980) and it can be imagined that infection with one serotype may confer some but not complete protection to reinfection with another serotype. It is not yet known how many human serotypes there are; presently there are three and there are likely to be more. Each reinfection with a "new" serotype would confer more immunological protection to the point where any subsequent rotavirus infections are largely asymptomatic. Rotavirus infection is not restricted to infants as originally thought, and not always is infection in adults asymptomatic. Von Bonsdorff, et al., (1978) reported an epidemic of rotaviral acute gastroenteritis in adults in Helsinki, Finland and in 1964 there was a rotavirus outbreak amongst 3,439 Truk islanders of all ages in the mid-Pacific (Foster, et al., 1980). Rotavirus has also been implicated in travellers diarrhoea (Bolivar, et al., 1978). With rotavirus circulating so readily in the adult population it can be envisaged that young adults, particularly parents, may be a major source of rotavirus for young children in which the disease is generally much more severe.

In this study, the fact that there were 132 specimens in total found to be positive for rotavirus antigen by RIA and ELISA using antiserum raised against SA_{11} rotavirus, indicates that there must be a relatively high degree of cross reactivity between all rotavirus types. Whether this cross reactivity has any neutralizing effect is not yet fully known. It may only be at one or two antigenic sites that differences resulting in

different serotypes may occur, and these may only be able to be discerned using very high dilutions of convalescent sera as used by Rodriguez, et al., (1978). Beards, et al., (1980) gave evidence that there may be at least five different human serotypes using serum neutralization tests but also showed a high degree of cross reactivity between these types. They suggested that two or more viral polypeptides may be involved in neutralization, one of which might be common to another serotype/s. The cross reactivity between animal and human isolates, and the incidence of rotavirus amongst animals in contact with young children prompted a closer look at the relationship of human and animal rotavirus, whether zoonotic infections may be occurring. An attempt to determine the relationship of human and animal isolates was made at the genetic level by electrophoresis and hybridization studies of the rotaviral dsRNA. These findings will be presented and elaborated on in the next two sections.

PART III: POLYACRYLAMIDE GEL ELECTROPHORESIS OF ROTAVIRAL DOUBLE-STRANDED RNA

PART III: POLYACRYLAMIDE GEL ELECTROPHORESIS OF ROTAVIRAL DOUBLE-STRANDED RNA

1. INTRODUCTION

Since its initial discovery in mice, rotavirus has been found to infect a wide variety of hosts. To date it has been isolated from mice, calves, piglets, foals, lambs, deer, cats, dogs, chickens, turkeys, monkeys, and man. Morphologically rotavirus isolated from all these hosts is indistinguishable. Serologically all isolates share a common group antigen but also show antigenic differences. Even within human rotavirus isolates a number of serotypes have been reported (Beards, et al., 1980; Rodriguez, 1978). Techniques such as RIA and ELISA using antiserum prepared against one particular isolate of rotavirus have been used in this study to detect rotavirus antigen in faecal specimens from a number of different host types (see Part II) reflecting the antigenic cross reactivity of these different isolates. The incidence of rotavirus amongst such a wide variety of host types and the frequent contact of man with these animals, has meant the possibility that rotavirus infection is a zoonotic disease had had to be seriously considered. One method to try and clarify the relationship of rotavirus isolates is to compare them at the genetic level. This section will develop the comparison of rotavirus isolates by "fingerprinting", using polyacrylamide gel electrophoresis (PAGE) of the rotaviral segmented, double-stranded RNA (dsRNA) genome.

Rotaviruses in common with the rest of the Reoviridae possess a segmented, dsRNA genome. Comparison by electrophoresis of dsRNA genomes has been used for: reoviruses of the three main serotypes (Hrdy, et al., 1975), orbiviruses (Gorman, 1979), and cytoplasmic polyhedrosis virus (CPV) (Payne and Rivers, 1976), to distinguish between serotypes, geographical distribution, and genetic change within a type. Such genetic comparison

has met with mixed success. Payne and Rivers (1976) were able to group CPV isolates according to their electrophoretic migration patterns. They found that isolates with very different electropherotypes were also different in their structural proteins and were serologically distinguishable. However, similarity in electropherotype does not necessarily mean one has identical virus isolates as shown in a later paper by Payne, et al., (1978) where two isolates, indistinguishable by RNA migration could be differentiated by antigenic and hybridization studies.

Hrdy, et al., (1979) found upon electrophoretic comparison of reovirus isolates, a striking polymorphism of migration of dsRNA segments within the three major serotypes. For types 1 and 3, the S_1 segment, coding for the haemagglutinin antigen, which is responsible for serotyping, was constant although the rest of the segments were variable in their electrophoretic migration. However, type 2 isolates showed polymorphism in all segments including the S_1 gene, which is consistent with the findings that reovirus type 2 isolates can be subdivided into a number of serological subtypes.

Similar studies have also been used in the comparison of the many different virus types that make up the orbivirus genus. In comparing 17 isolates of Wallal group viruses, Gorman (1979) showed that use of PAGE profiles of RNA alone were inadequate for the classification of these viruses into subgroups. He pointed out that co-migration of RNA segments indicates similarity in molecular weight and does not indicate the relationship between segments.

The electrophoretic characterization of reoviruses, orbiviruses, CPV's and influenza viruses has aided in the understanding of the genetic variability of these viruses and has helped in following the epidemiological impact of different electropherotypes in host populations. Genomic variability may be reflected in antigenic variability in some cases and this may

effect the host range or virulence of the virus. It was of interest therefore, with so many host species being infected with rotavirus, to characterize the various isolates by PAGE of the dsRNA genome and see if there was any apparent relationship of electropherotype with epidemiological patterns.

A number of questions were asked as the electropherotypes of a number of isolates were compared:

1) How much variability exists within a series of isolates from a single host species?

2) How much variability exists between isolates that originate from different species?

3) Do isolates within a single outbreak show variability?

4) Is there any evidence of consistent patterns defined by geographical areas?

5) Can the dsRNA pattern be used to reliably predict the host source of the isolate?

6) Are there any electropherotypes common to more than one host species?

Of particular interest is the comparison of isolates from animals that have a high contact rate with young children. If rotavirus infection has a zoonotic spread then rotavirus in cats and dogs would be of prime interest.

At the time of the outset of this study only limited comparisons of rotaviral genomes by PAGE had been made. Rodger, et al., (1975) had characterized the genome profile of a calf rotavirus isolate; Todd and McNulty (1976), porcine rotavirus; Verly (1977) showed the existence of more than one calf electropherotype present in France. In 1976, Schnagl and Holmes showed that a human and calf rotavirus isolate had different electropherotypes as did Kalica, et al. Within New Zealand a calf and

human rotavirus electropherotype was resolved in 1977 (Schroeder, Honours Thesis). However, the question still remained as to the overall variability of rotavirus genome electropherotypes and whether there were well defined, host related, electropherotypes? It was also of interest to see whether an electropherotype of an isolate from one host species, say a cat or dog, could also be found in another host species such as man.

Because rotaviruses have a segmented genome it is theoretically feasible that if one host animal were to be infected with two different rotavirus types at any one time that there may be interchange of genetic material and a hybrid of the two types result in a "new" type. It would be of interest to see then by PAGE whether any patterns are revealed that indicate that such an event has taken place. Since the start of this study the reassortment of two rotaviral genomes has been achieved in vitro using two tissue culture adapted strains, SA_{11} and neonatal calf diarrhoea virus (NCDV) (Matsuno, et al., 1980).

Over the period of this study a number of papers have been published on electropherotypes of rotaviral isolates; these will be discussed in the context of the conclusions from this study.

In the rest of this chapter will be discussed the methods of dsRNA purification from faecal specimens, and electrophoretic separation of genome segments on polyacrylamide slab gels. The electropherotypes of a number of human and animal isolates will be shown and their relevance discussed. The electropherotypes of cat and dog rotaviruses are presented which to date have not been published by any other investigators.

2. MATERIALS AND METHODS

2.1 Purification of Rotaviral dsRNA

Faecal specimens that had been found to be positive by ELISA for rotavirus were extracted for dsRNA by the method of Croxson and Bellamy (1981). Faeces were suspended (10-20%) in Tris-acetate buffer (0.1 M, pH 8.0). Cold lithium dodecyl sulphate was added to 1% and the suspension was left for 15-30 minutes at 0 °C. Solid faecal material was removed by low speed centrifugation (10,000 g for 15 minutes) and viral dsRNA pelleted by ultracentrifugation at 120,000 g for 60 minutes. The pellet was resuspended in electrophoresis sample buffer (see Appendix I, 7.5) and stored at -70 °C until used in electrophoretic experiments. Immediately prior to application on polyacrylamide gels, samples were heated to 80 °C for 2 minutes.

An alternative method that was used initially for dsRNA extraction was by using hot phenol. Rotavirus containing faeces were suspended in 10 ml of 0.01 M sodium acetate buffer, pH 5.0, 0.05 M sodium chloride and 0.1 g of sodium lauryl sulphate. To this suspension was added 10 ml of 90% (v/v) phenol at 60 °C. The mix was incubated at 60 °C for 3 minutes with shaking every 30 seconds. The extract was cooled on ice and the aqueous phenol phase separated by centrifugation at 4,000 g for 5 minutes. The aqueous phase was removed and re-extracted twice more with 6 ml and 4 ml of 90% phenol, as above. The dissolved phenol was removed from the final aqueous phase by extraction 3-5 times with ether (4 °C). The ether phase was discarded. The dsRNA was precipitated by adding 2 volumes of sodium chloride saturated ethanol. After storage at -20 °C overnight, the RNA was pelleted by centrifugation at 5-7,000 g for 10 minutes. When a visible precipitate could not be seen the RNA was pelleted by ultracentrifugation at 160,000 g for 30 minutes. The dsRNA pellet was washed with absolute

ethanol and then dissolved in electrophoresis sample buffer. The dsRNA was relatively pure by this method and an estimate of the concentration could be made by a spectrophotometric reading at 260nm and 280nm. The 260:280 ratio of pure RNA should be 2.0 or greater. A reading of 1 absorbance unit at 260nm represents 40µg per ml of RNA. The RNA was stored at -70°C until used in electrophoresis experiments.

2.2 Polyacrylamide Gel Electrophoresis (PAGE) of Rotaviral dsRNA

The electrophoretic system used was essentially the discontinuous system of Laemmli (1970) used for study of bacteriophage T_4. Virion RNA segments were resolved by electrophoresis for 6-9 hours at 120 V on 10% polyacrylamide slab gels using apparatus designed and made in the laboratory based on the designs of Poyton (1978) and Driedger and Blumberg (1978). A 5% stacking gel was used and the RNA was applied to the wells at a loading of 0.5-5µg of RNA in electrophoresis buffer (see Appendix I for buffers and gel recipe used).

The slab gels were made by taking two clean glass plates, inserting plastic spacers between them (0.5mm thick) and taping the sides and bottom with stretchable plastic tape. A freshly made solution of 10% polyacrylamide was poured between the two plates to a level 2cm from the top. This was then overlaid with 2mm of distilled water, prior to polymerization of the acrylamide, to ensure an even surface. The plates were kept vertical until the gel had set (20-30 minutes). The water was poured off and freshly made 5% polyacrylamide solution was poured to the level of the top of the two plates. Immediately the plastic well former was inserted so that the gel polymerized round it. After polymerization (30 minutes) the well former was carefully removed and any loose polymerized acrylamide was washed out of the wells. The tape was removed from the base of the two plates which were then placed in the lower electrophoresis tank filled

with Tris-glycine electrophoresis buffer. The top tank which was sealed onto one of the glass plates was filled with electrophoresis buffer. Samples to be electrophoretically characterized were layered into the wells using a syringe needle. Immediately all samples had been added to the slab gel, 60 volts was applied for 15 minutes and then increased to 120 volts for the time required. After electrophoresis the gel was carefully removed from the plates and visualized with ethidium bromide (1μg per ml) for 30 minutes or overnight and then visualized by UV light on a chromato-Vue ultra-violet transilluminator screen and photographed with Kodak Tri-x-pan film.

3. RESULTS

3.1 Purification of Rotaviral dsRNA

Of the two methods used for purification of rotaviral dsRNA from faecal material, the detergent method of Croxson and Bellamy (1981) proved to be the most rapid for handling a number of samples and appeared to be relatively sensitive. By this method however, dsRNA samples were never as pure as with the hot phenol method. This method resulted in blurred electropherograms and may have been a cause of some of the double imaging of bands seen at various times. If a sample still has a high degree of protein present it can be imagined that instead of the dsRNA moving directly into the polyacrylamide gel, it may slowly "leach" out of the protein material to which it binds and so give a blurred "fingerprint" rather than sharply resolved bands.

The phenol purification method was more time consuming to perform and because of the number of manipulations involved, only two or three specimens could be efficiently handled at any one time. However, this method did give a relatively pure yield of dsRNA. It was a less sensitive method than by using detergent extraction with probably higher percentages of the dsRNA being lost during the various manipulations.

Double-stranded RNA, extracted by the detergent method could be further purified by phenol if electrophoresis proved difficult. It was also found that isoamyl chloroform (9 volumes chloroform:1 volume isoamyl alcohol) could clarify detergent extracted dsRNA by adding an equal volume of this to the dsRNA, mixing, separating the two phases by low speed centrifugation and removing the isoamyl chloroform phase. The inclusion of a sonication step at the initial stages was also found to increase the yield of dsRNA for both extraction procedures.

3.2 Rotaviral dsRNA Electropherotypes

The electropherotypes of rotaviral dsRNA preparations were made for a total of 48 different isolates. Of these 30 were for human isolates, 9, calf; 1, cat; 6, dogs; and 2 for foals. From these 48 isolates typed by electrophoresis, 15 distinct electropherotypes were resolved: 7, human; 5, calf; 1, cat; 1 dog and 1 foal. Four cultivable rotaviruses were also electropherotyped (NCDV, SA_{11}, WA (human), and NI (calf)).

By comparing electropherotypes resolved from different experiments with common types an overall composite of the different types was able to be drawn to facilitate better visual comparison (Fig. 20). The composite is not an exact representation of all the different types because there were differences in electrophoresis running times for different experiments and correction values could only be estimated. However, the composite does reveal some of the apparently major differences between different isolates. The electropherotypes have been grouped according to the host type from which the isolate was derived, and the different electrophoretic profiles classified under different groupings. For example, there are seven different human electropherotypes, for type A (HU-A) there are 15 different isolates with this profile. Some of the electropherotype characteristics are summarized in Table 11.

There were some isolates that were unable to be typed due to a lack of clear resolution of bands upon electrophoresis. Actual electropherograms with representatives of some of the different electropherotypes are shown in Figures 14 to 20.

Fig. 13--Rotaviral dsRNA Migration by PAGE. Approximately 1µg dsRNA per channel. 120 volts for 8 hours. Ethidium bromide stained (1µg per ml) and UV visualized.

HU = human, CN = Canine, DUN = Dunedin.

e.g., HU/DUN/8/79 = human rotavirus/Dunedin/specimen number 8/year 1979 (Rodger and Holmes, 1979). Note migration differences between human specimen numbers 46, 143 and 151 and canine specimen.

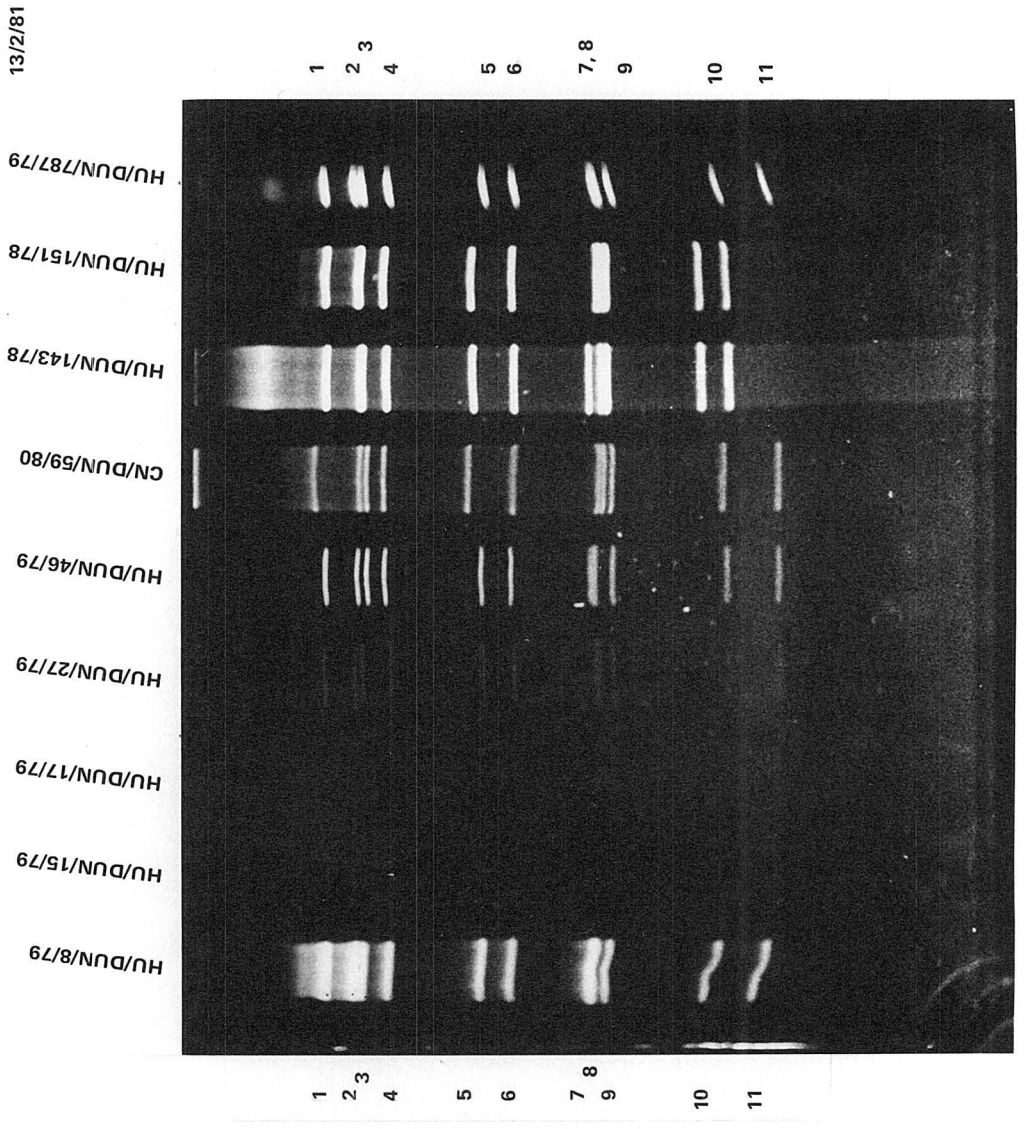
13/2/81
1
2 3
4
5
6
7,8
9
10
11
HU/DUN/787/79
HU/DUN/151/78
HU/DUN/143/78
CN/DUN/59/80
HU/DUN/46/79
HU/DUN/27/79
HU/DUN/17/79
HU/DUN/15/79
HU/DUN/8/79
1
2 3
4
5
6
7 8
9
10
11

Fig. 13

Fig. 14--Rotaviral dsRNA Migration by PAGE. Approximately 1μg dsRNA per channel, 120 volts for 8 hours. Ethidium bromide stained (1μg per ml) and UV visualized.

HU = human, NCDV = neonatal calf diarrhoea virus, DUN = Dunedin. e.g., HU/DUN/8/79 = Human rotavirus/Dunedin/specimen number 8/year, 1979. Note migration differences between specimen numbers 79, 88, 89 and 151.

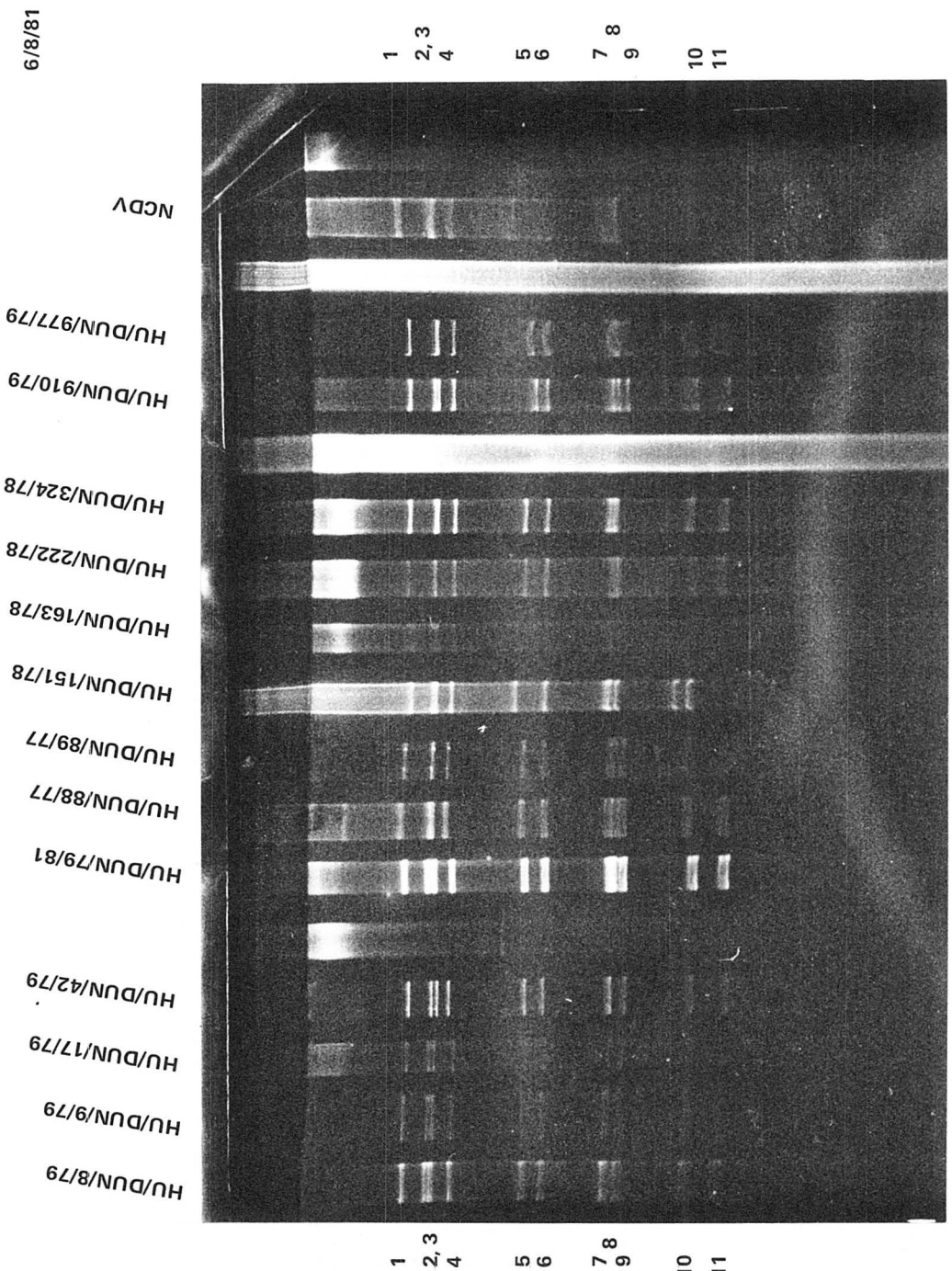
6/8/81
1
2,3
4
5
6
7
8
9
10
11
NCDV
HU/DUN/977/79
HU/DUN/910/79
HU/DUN/324/78
HU/DUN/222/78
HU/DUN/163/78
HU/DUN/151/78
HU/DUN/89/77
HU/DUN/88/77
HU/DUN/79/81
HU/DUN/42/79
HU/DUN/17/79
HU/DUN/9/79
HU/DUN/8/79

Fig. 14

Fig. 15--Rotaviral dsRNA Migration by PAGE. Approximately 1µg dsRNA per channel, 120 volts for 8 hours. Ethidium bromide stained (1µg per ml) and UV visualized.

BO = bovine, HU = human, DUN = Dunedin

e.g., BO/DUN/25/79 = Bovine rotavirus/Dunedin/specimen number 25/year 1979. Note migration differences of human specimens 9 and 7.

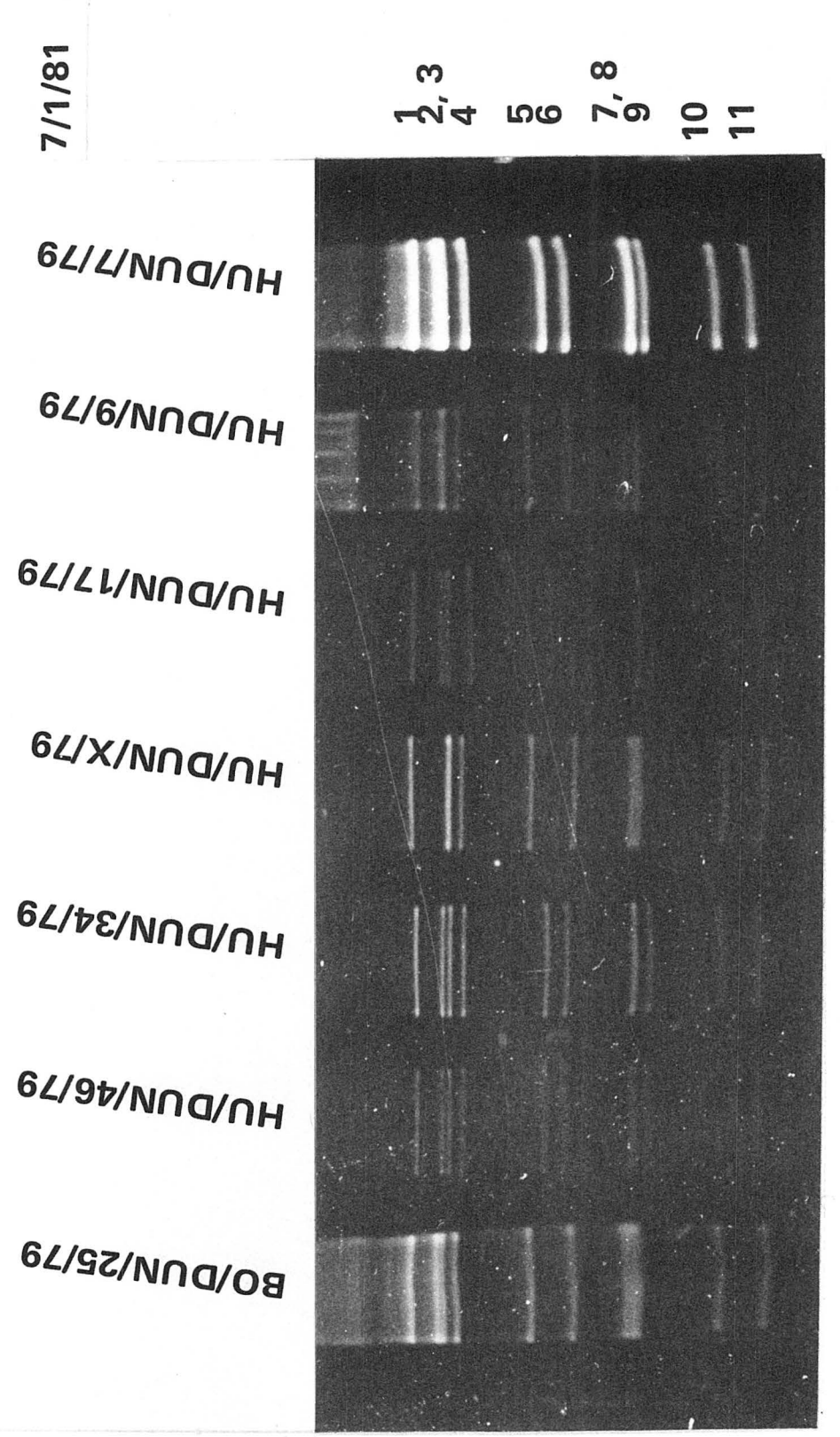
7/1/81
1, 2, 3, 4
5
6
7, 8, 9
10
11
HU/DUN/7/79
HU/DUN/9/79
HU/DUN/17/79
HU/DUN/X/79
HU/DUN/34/79
HU/DUN/46/79
BO/DUN/25/79

Fig. 15

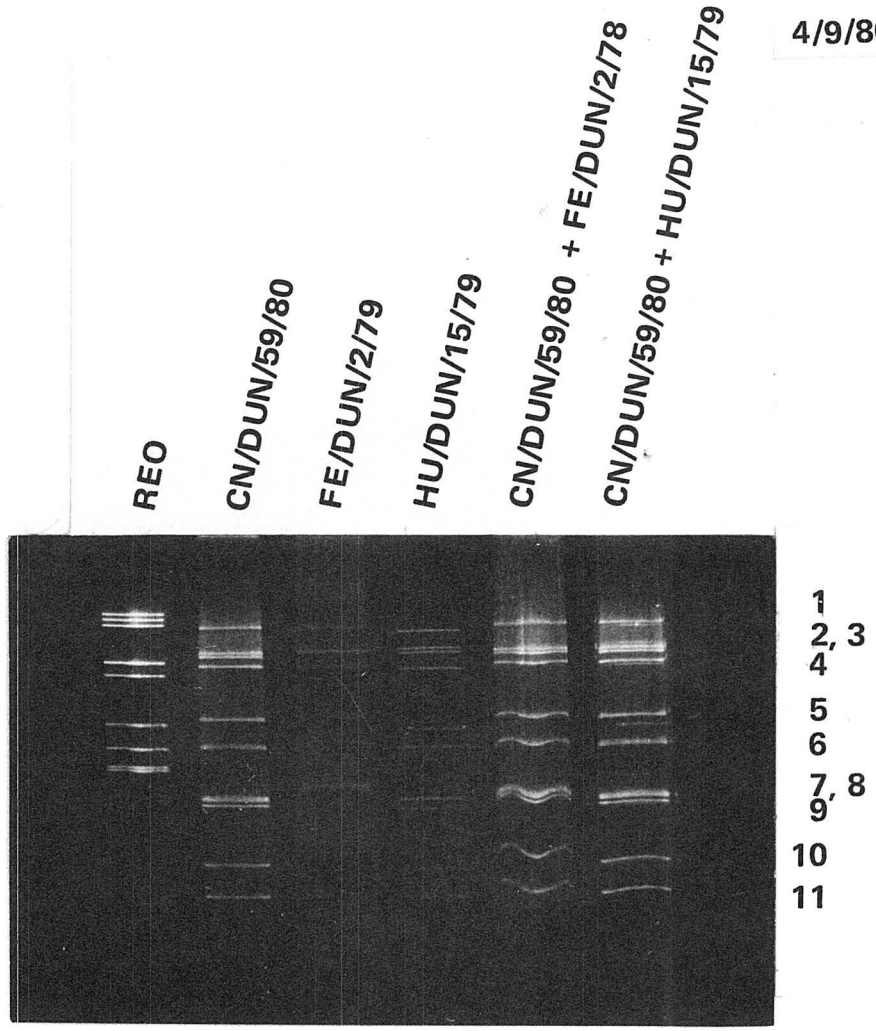

Fig. 16--Rotaviral dsRNA Migration by PAGE. (1µg dsRNA per channel, 120 volts for 8 hours). Ethidium bromide stained (1µg per ml) and UV visualized.

REO = reovirus type 3, CN = canine, FE = feline, HU = human. The 5th and 6th channels were co-electrophoresis of 2 specimens.

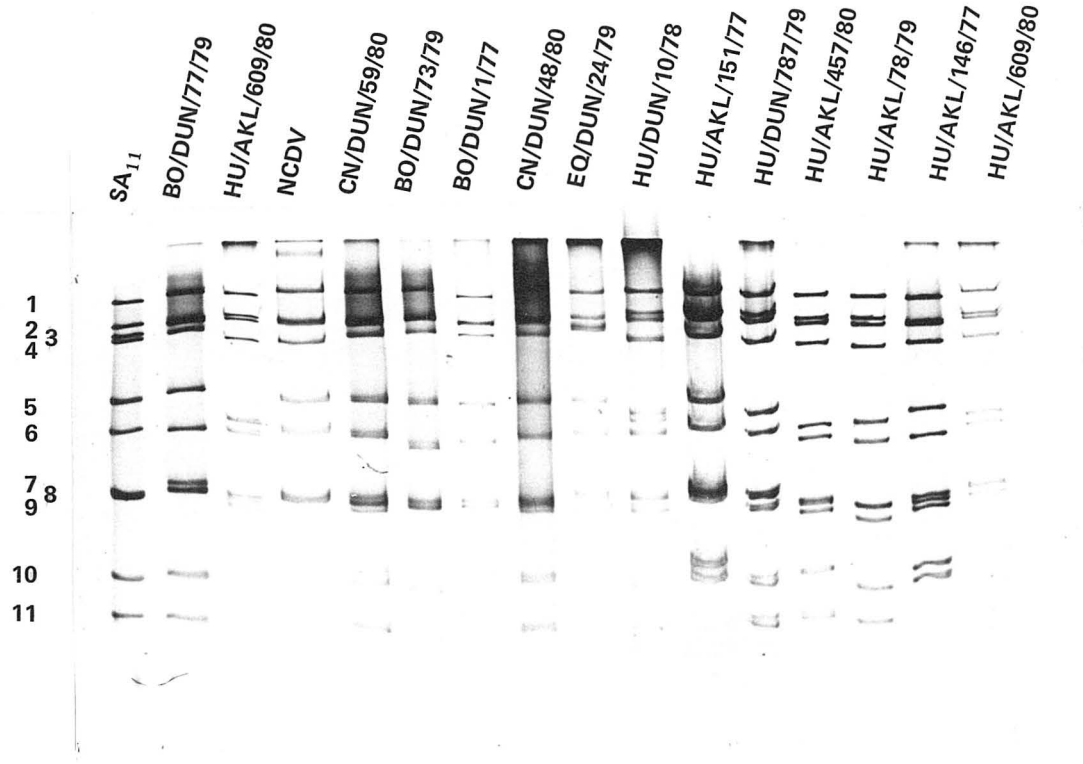

Fig. 17--Rotaviral dsRNA Migration by PAGE. (1µg dsRNA per channel, 120 volts for 8 hours). Ethidium bromide stained (1µg per ml) and UV visualized.

SA = Simian, BO = bovine, NCDV = neonatal calf diarrhoea virus, HU = human, CN = canine, EQ = equine, DUN = Dunedin, AKL = Auckland. Note HU/DUN/10/78 which has an extra band between 5 and 6. This may indicate the presence of two rotavirus types in one host.

Fig. 18--Rotaviral dsRNA Migration by PAGE. (1μg dsRNA per channel, 120 volts for 8 hours). Ethidium bromide stained and UV visualized.

HU = human, CN = Canine, BO = bovine. Note migration differences between bovine isolates 70 and 71

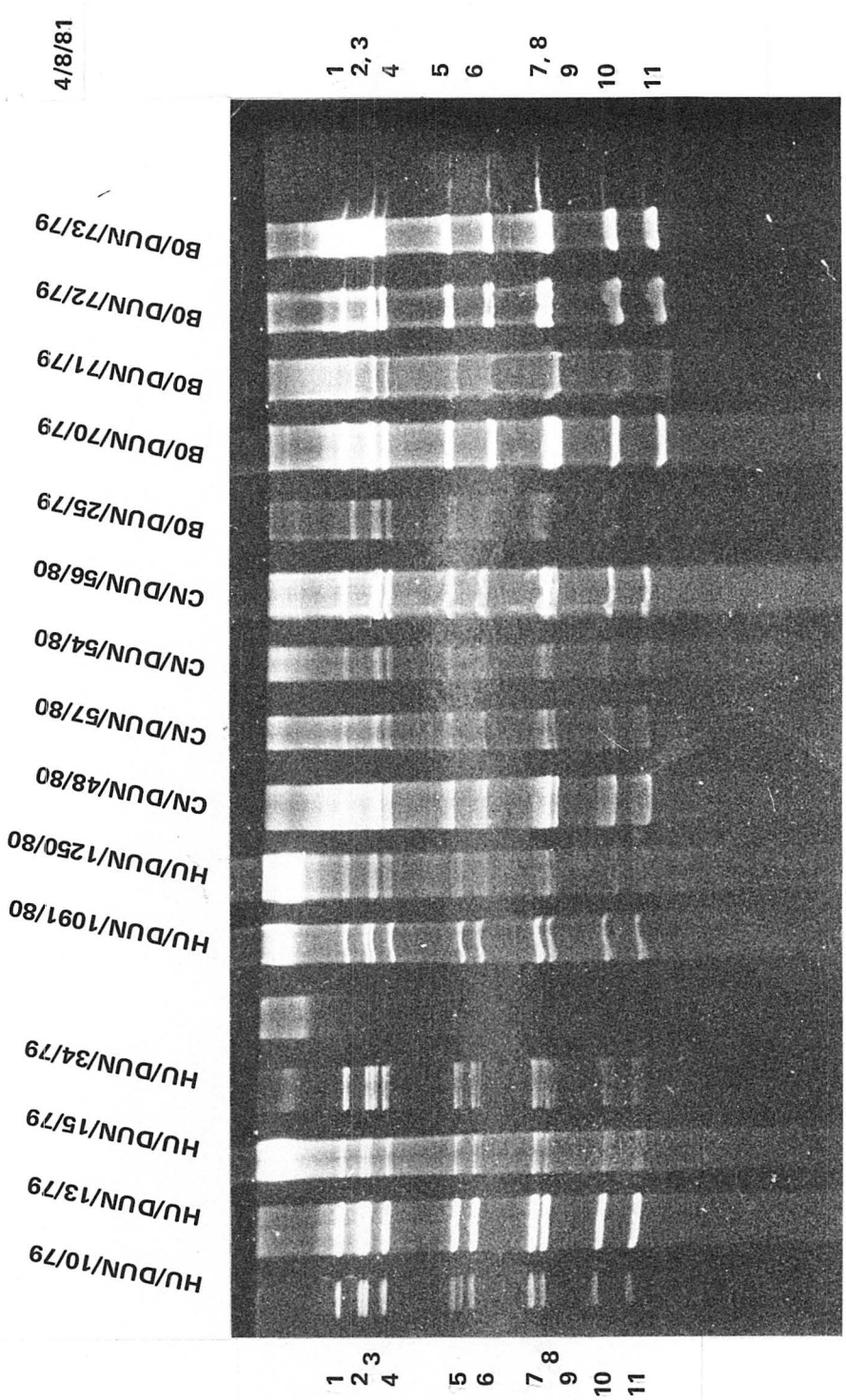
4/8/81
1
2, 3
4
5
6
7, 8
9
10
11
B0/DUN/73/79
B0/DUN/72/79
B0/DUN/71/79
B0/DUN/70/79
B0/DUN/25/79
CN/DUN/56/80
CN/DUN/54/80
CN/DUN/57/80
CN/DUN/48/80
HU/DUN/1250/80
HU/DUN/1091/80
HU/DUN/34/79
HU/DUN/15/79
HU/DUN/13/79
HU/DUN/10/79
1
2
3
4
5
6
7
8
9
10
11

Fig. 18

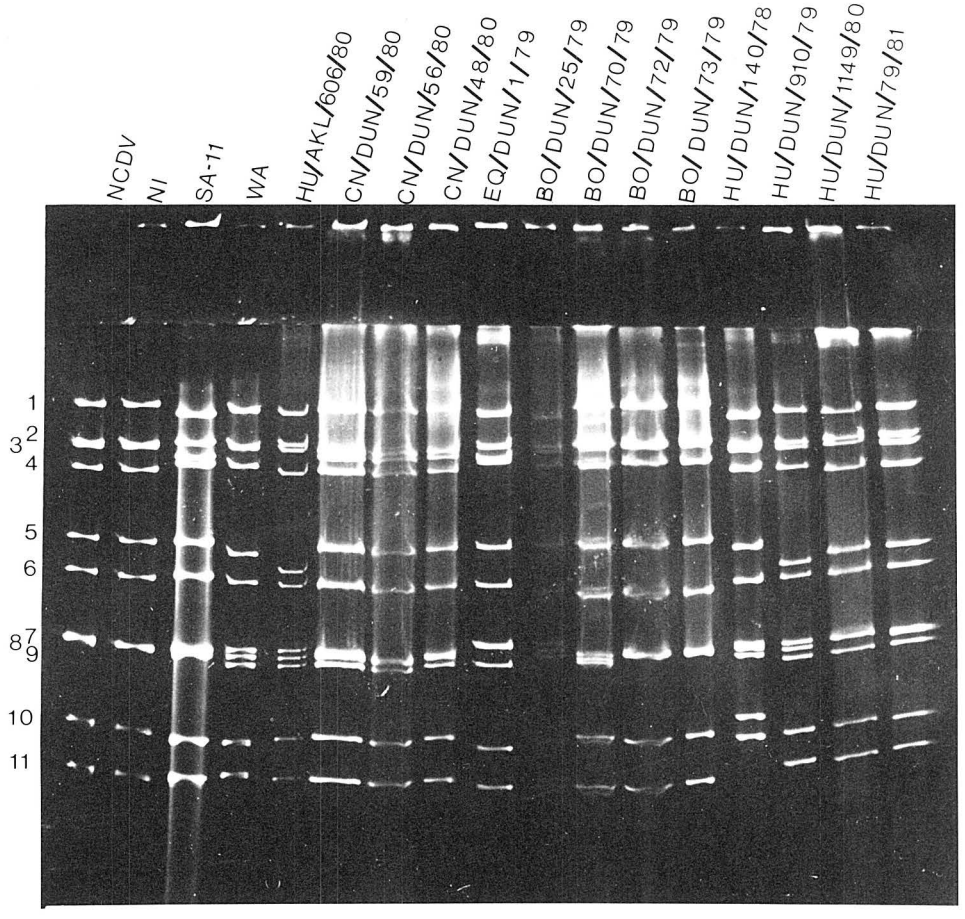

Fig. 19--Rotaviral dsRNA Migration by PAGE. (1μg dsRNA per channel, 120 volts for 8 hours). Ethidium bromide stained and UV visualized.

NCDV = neonatal calf diarrhoea virus, NI - Northern Ireland calf virus, SA_{11} = simian virus, WA = cultivable human virus, HU = human, CN = canine, BO = bovine, EQ = equine, DUN = Dunedin, AKL = Auckland. Note migration differences between bovine isolates 25, 70 and 7s and human isolates 140, 910 and 1149.

Fig. 20--Composite of Rotaviral Electropherotypes Resolved. HU = human, BO = bovine, FE = feline, CN = canine, EQ = Equine, NI = Northern Ireland calf virus, NCDV = neonatal calf diarrhoea virus, SA_{11} = simian, WA = cultivable human virus.

Examples of Different Electropherotypes

HU-A	HU/DUN/46/79	(Fig. 13, channel 5)
B	HU/DUN/X/79	(Fig. 15, channel 4)
C	HU/DUN/143/78	(Fig. 13, channel 7)
D	HU/DUN/151/78	(Fig. 13, channel 8)
E	HU/DUN/88/77	(Fig. 14, channel 7)
F	HU/DUN/324/78	(Fig. 14, channel 12)
G	HU/DUN/910/79	(Fig. 14, channel 14)
BO-A	BO/DUN/18/79	(not shown)
B	BO/DUN/25/79	(Fig. 19, channel 10)
C	BO/DUN/70/79	(Fig. 19, channel 11)
D	BO/DUN/73/79	(Fig. 19, channel 13)
E	BO/DUN/77/79	(Fig. 17, channel 2)
FE-A	FE/DUN/2/79	(Fig. 16, channel 3)
CN-A	CN/DUN/59/80	(Fig. 16, channel 2)
EQ-A	EQ/DUN/24/79	(Fig. 17, channel 9)

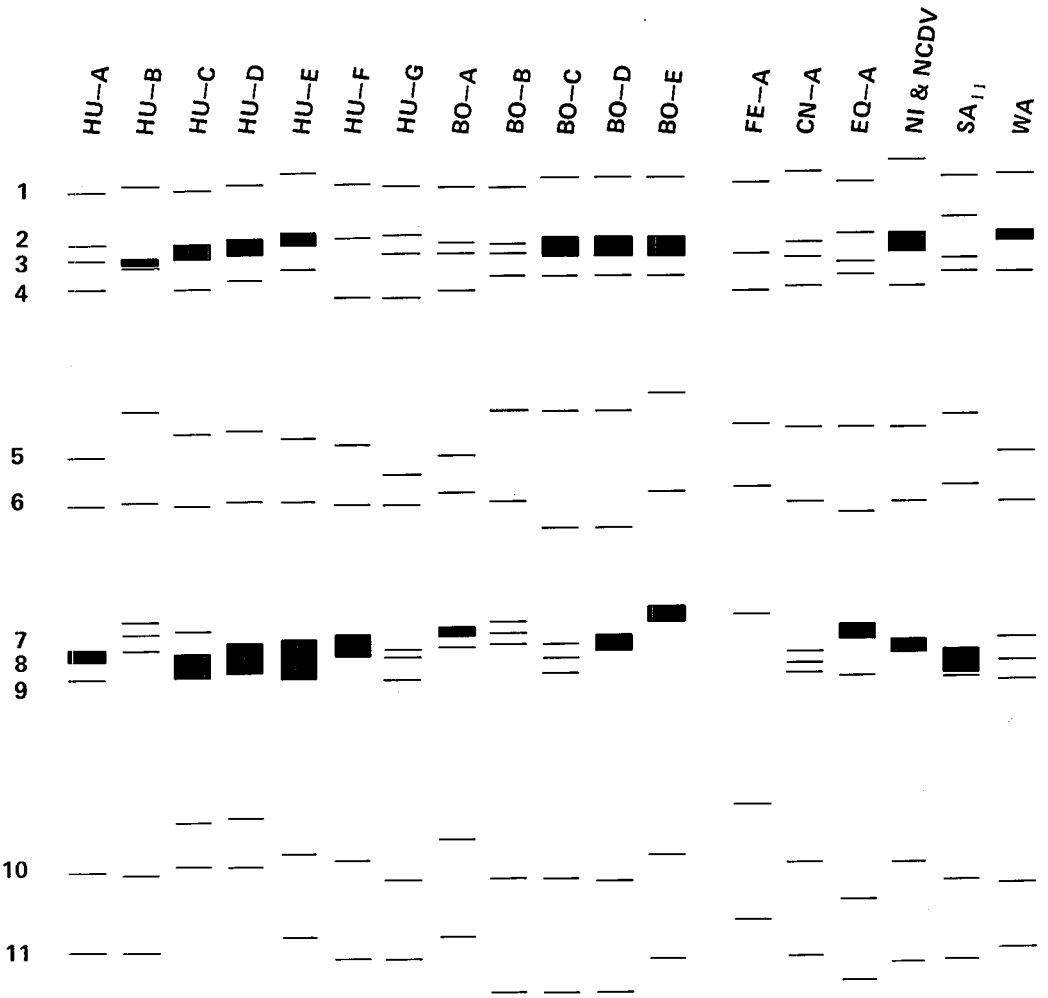
HU–A
HU–B
HU–C
HU–D
HU–E
HU–F
HU–G
BO–A
BO–B
BO–C
BO–D
BO–E
FE–A
CN–A
EQ–A
NI & NCDV
SA11
WA
1
2
3
4
5
6
7
8
9
10
11

Fig. 20

TABLE 11

Rotaviral Electropherotype Characteristics

Electropherotype	Number of Bands Resolved	Number of Isolates of This Type	Segments That Migrate Together	Similar Electropherotypes
HU-A	10	15	7 and 8	HU-G
B	10	1	2 and 3	BO-B
C	9	2	2 and 3; 8 and 9	HU-D
D	8	2	2 and 3; 7, 8 and 9	HU-B, E, NI
E	8	2	2 and 3; 7, 8 and 9	HU-D
F	8	3	2 and 3; 7, 8 and 9	---
G	11	1	---	CN-A
BO-A	10	2	7 and 8	NI, NCDV
B	11	1	---	HU-B, EQ-A
C	10	1	2 and 3	BO-D
D	8	3	2 and 3; 7, 8 and 9	BO-C
E	8	1	2 and 3; 7, 8 and 9	BO-D, NCDV
FE-A	8	1	2 and 3; 7, 8 and 9	---
CN-A	11	6	---	HU-G
EQ-A	10	1	7 and 8	BO-B
NCDV	8	1	2 and 3; 7, 8 and 9	NI
NI	8	1	2 and 3; 7, 8 and 9	NCDV
SA_{11}	10	1	7 and 8	---
WA	10	1	2 and 3	HU-E

HU = human, BO = bovine, FE = feline, CN = canine, EQ = equine, NCDV = neonatal calf diarrhoea virus, SA_{11} = simian, WA = cultavable human isolate.

4. DISCUSSION

As shown by the results of this section of electrophoretic migration of rotaviral dsRNA, the rotaviruses form a very heterogeneous group when viewed at the genetic level. From 48 isolates electropherotyped, 15 distinct patterns were resolved, i.e., just about every fourth isolate is different. PAGE is not a particularly sensitive technique for determining genetic differences but will only resolve differences of a relatively large molecular weight. Therefore, even patterns that appear to be similar may in fact be different when viewed at the level of base sequences. Another important factor that needs to be kept in mind when doing electrophoretic comparisons is that differences in migration pattern between isolates may be insignificant in the context of the protein structure of the virus, its antigenicity, infectivity and virulence. The significance of such genetic differences needs to be interpreted in the light of the epidemiological patterns of the virus.

It has been shown that locally there are at least 15 different electropherotypes and yet all of these isolates were able to be detected at the antigenic level with one specific antisera prepared against SA_{11} rotavirus. This implies that there are still some points of antigenic similarity between them all. It is possible that the antisera may have been detecting more than one antigen and that these antigens may not all be common to all the isolates but enough are common to cause cross reactivity. Of course, not all the isolates would necessarily possess the same set of cross reactive antigens. Whether this antigenic cross reactivity is important in infectivity and virulence is not known but it does tell us that the heterogeneity shown at the genetic level is not the full picture in itself.

In the introduction to this chapter a number of questions were outlined to help evaluate the significance of variation in electropherotypes:

1) How much variability exists within a series of isolates that originate from a single host species?

2) Do isolates within a single outbreak show variability?

3) How much variability exists between isolates that originate from different species?

4) Is there any evidence of consistent patterns defined by geographical areas?

5) Can the dsRNA pattern be used to reliably predict the host source of the isolate?

6) Are there any electropherotypes common to more than one host species?

Firstly, the variability of electropherotypes of isolates from a single species is shown to be high. In the two host species where there was a significant number of isolates typed (human and bovine) there was shown to be a high level of intra-species heterogeneity. This was not just a reflection of seasonal or geographical sampling variation. For example, in the human isolates the predominant type HU-A, was first resolved in 1978 when it was the dominant type and was found right through into 1981. At the same time and from the same human populations (the Dunedin urban area) there were a number of other electropherotypes being resolved. In 1978 alone there were four different types present out of a total of eight isolates typed. Amongst the bovine isolates the same situation occurred. All isolates were collected within the one year, 1979, and yet there were 5 different electropherotypes resolved. Some of these isolates were from the North Island and so geographical separation may account for some of the variation. However, two of the isolates were collected from two different farms on the same day, within ten miles of each other and yet were electrophoretically quite dissimilar (BO-A and BO-B).

Secondly, just as there is a high degree of intra-species variation so there is a high degree of inter-species variation. The variation at the intra-species level appears to be as great as at the inter-species. This variation is so great and spread over all the different segments, that it is not possible to detect a consistent pattern defined by geographical areas or to reliably predict the host source of the virus (questions 4 and 5).

The answer to the third question is not so clear. Within a single outbreak of rotavirus in a litter of greyhound pups the electropherotype resolved from all the different isolates was consistent throughout showing no variation when collected from different individuals or at different times. The virus in this case underwent no changes during the outbreak and there was only one type involved. However, during a winter outbreak of gastroenteritis in Dunedin in 1979, when a number of children were hospitalized, there were three different electropherotypes resolved. Of all the isolates typed, 77.8% were from type HU-A. It appears that there may be a predominant type present during an outbreak that infects the majority of hosts with a number of other types playing a minor role in infections in the community. These other types may be important in the variability of the virus over different seasons. Through immunogenic pressure or recombination between types a new type may become predominant within a population over a period of time. Espejo, et al., (1980) found that between 1977 and 1978 there was a distinct change in the proportion of two human electropherotypes present in the population. Both types were present throughout the time period but one type replaced the other as the predominant type over that period. They divided the human electropherotypes into two main groups; however, within these groups small differences could be observed in segments 7 to 9. Such a division could be made for the human types in this present study in that there are two types that show

"short" patterns (HU-C and HU-D) and the rest show "long" patterns. However, the types would be very heterogeneous even within these two groups. The significance of the "short" pattern as compared with the "long" pattern is not known and it is interesting to note that to date no "short" patterns have been resolved for animal rotavirus isolates locally or, according to the literature, elsewhere. In a similar type of study to that done here but over a much longer time period (1973-1979) and a greater number of specimens (116), Rodger, et al., (1981) in Melbourne, Australia found a total of 17 different human types. More importantly they found there was a sequential pattern of appearance, with a limited number of electropherotypes present at any one time. There needed to be a greater number of human isolates electropherotyped in the present study and for a greater time period to be able to make any assessment of possible trends or progressions in electropherotype predominance and transition.

Co-electrophoresis of two isolates to give an assessment of similarity has been tried by a number of groups with mixed success (Rodger, et al., 1981; Espejo, et al., 1980; Todd, et al., 1980; Rodger and Holmes, 1979; and Kalica, et al., 1978). As is particularly evident in the papers by Rodger and Holmes (1979), Kalica, et al., (1978) and Todd, et al., (1980) the results obtained from such comparisons is not always what is expected. For instance, in Kalica, et al., (1978) the results in co-electrophoresis of a United Kingdom calf isolate (UK) and SA_{11}, segment differences were observed at: 2, 3, 4, 5, 6 and 9 (UK vs SA_{11}). When UK was co-electrophoresed with OA isolated from intestinal washings of sheep and cattle in South Africa differences were observed in segments: 2, 4, 5 and 6 (UK vs DA).

From these two results it can be predicted that when OA and SA_{11} are co-electrophoresed there should be differences observed in segments 3 and 9 and possibly in 2 to 6. In fact, the result obtained shows differences

in segments 3 and 5 only. The inconsistency of predicted results with those actually obtained occurs time and again in these papers and probably reflects the inability of the system to detect minor differences in electrophoretic migration. Co-electrophoresis experiments were not routinely done in this study as the mentioned problems of inconsistencies indicated that a more sensitive technique was needed for direct genetic comparison.

One further question was asked regarding the comparison of different electropherotypes and their relationship to host species; that is, are there any electropherotypes common to more than one host species? From this study there is no direct evidence of rotavirus types being able to infect more than one host species. However, there are some electropherotypes from different species that appear to be very similar and at least as similar as other related species types. For instance, type BO-B is more like the human type HU-B than the other bovine types. The only canine electropherotype, CN-A, is very similar to the human type HU-G. This could be significant considering the two types were isolated in the same year in the Dunedin urban area.

It appears from this study and many others that have been done that there are a very large number of electropherotypes for rotavirus irrespective of the host species. The intra-species differences appear to be as great as inter-species and that the majority of segments are involved in this variability (segments 1, 6 and 10 appear to be the most constant). If the pool of genetic material is so large and recombination of genetic segments occurs in nature as has been shown *in vitro* (Matsuno, *et al.*, 1980) then it is to be expected that isolates from different species may share common dsRNA segments as evidenced by similar electrophoretic mobility and also retain segments of differing mobility. Hence, it is unlikely in a study such as this, with a relatively small number of isolates typed, to expect to find identical electropherotypes from different host species.

Although PAGE of rotaviral isolates showed there are a large number of different types when compared at the genetic level, much more than indicated by serological and epidemiological patterns, the technique gave no indication of the degree of dissimilarity. Neither could it tell if 2 segments from different isolates that migrated the same distance were in fact genetically the same or whether it was just a molecular weight similarity. To determine more fully the degree of similarity or dissimilarity of rotaviral dsRNA from different isolates, molecular hybridization of labelled DNA copies of rotaviral RNA with rotaviral RNA of another isolate was used as described in the next section.

PART IV: MOLECULAR HYBRIDIZATION

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1. INTRODUCTION

From polyacrylamide gel electrophoresis of rotaviral dsRNA it is noticeable that there is a common overall pattern of RNA migration and yet there is also a large amount of variability in migration patterns between different isolates (see Part III). Hybridization of rotaviral RNA from different isolates was used as a means to determine more precisely the degree of homology between these isolates. In this section the general applications and principles of molecular hybridization will be outlined and particularly the methods and results of rotaviral RNA hybridization.

1.1 Applications of Hybridization Technology

During the 1960's and '70's an increased understanding of the function of DNA and RNA in cellular functions and in the mechanisms of replication of genetic material have led to a number of developments in the study of viral and cellular genetic material and its relationship to specific proteins and functions. The ability to sequence, translate, splice, clone and hyridize genes has led to a greater ability to explore the nature of molecular events within the cell, and relationship at the genetic level, of different animal species, bacterial serotypes, and different viral isolates. Hybridization techniques have been of value in determining the degree of relationship of isolates of particular viruses from different host species; for example, tymo viruses (Kummert, et al., 1978), papillomaviruses (Law, et al., 1979), polyomaviruses (Howley, et al., 1979), cucumber mosaic virus (Gould and Symons, 1977), and reovirus (Bellamy and Joklik, 1967).

Law, et al., (1979) in their studies on papillomavirus were able to show that there was conservation of up to 70% of the genetic material among

all the genomes of the papillomaviruses tested. They also were able to add further evidence to the distinct nature of the papilloma and polyoma virus genera. Isolates of the papillomavirus genus produced similar symptoms in their natural hosts (humans, cattle, rabbits, dogs, sheep, chaffinches, horses and others). These hybridization experiments were able to deduce more precisely the degree of relationship between isolates.

Howley, et al., (1979) using similar methodology to Law, et al., (1979) showed that there is some sequence homology between the antigenically distinct polyomaviruses - simian virus 40, human papovavirus and polyomavirus. They concluded that all members of the polyomavirus genus are related evolutionarily and that certain regions of the genomes have retained similarity in their nucleic acid sequence. This genetic similarity was not always detectable at the antigenic level. The partial homology detectable by hybridization techniques was later verified by sequencing of the polyoma and the SV_{40} genomes.

1.2 Principles of Molecular Hybridization

Techniques for hybridization of genetic material in order to determine degrees of homology were largely developed from two findings in 1960 and 1961. Firstly, it was reported that DNA could be dissociated into two strands and the physical properties and biological activity of double-stranded DNA could then be restored by incubation under appropriate conditions. Secondly, virus specific RNA, made by bacteria during viral infection, was shown to pair with the viral DNA (Britten and Kohne, 1968). These two findings indicated that genetic material from different sources could be mixed, allowed to hybridize and, according to the reaction conditions, the degree of homology be determined.

Techniques were developed for the immobilization of single-stranded DNA in cellulose, agar and nitrocellulose filters. This made it possible

for the hybridization of radioactively labelled, single-stranded fragments of DNA or RNA, to the immobilized DNA to be assayed (Britten and Kohne, 1968).

A further development in the study of the structure of DNA was the discovery that a restriction endonuclease from Haemophilus influenzae makes double-stranded breaks at specific sequences in DNA. Other enzymes with similar properties have since been discovered (Southern, 1975). This has allowed the comparison by hybridization of specific portions of genetic material. Fragments produced by these enzymes can be separated with high resolution by electrophoresis in agarose and polyacrylamide gels. Initially, for hybridization studies of individual fragments the gels were sliced, the DNA eluted and hybridized to RNA/DNA either in solution or after binding the DNA to filters. This method was time consuming and led to loss of resolution. In 1975, Southern developed a method for transfer of DNA fragments separated by electrophoresis, direct from the gel to strips of cellulose nitrate. These strips could then be used in hybridization experiments. This came to be known as the "Southern Blot" technique. The technique has played an important part in advances in analyzing and purifying many DNA fragments. However, there are some limitations with the technique: (1) large restriction fragments are not transferred from agarose gels efficiently and very small fragments (less than 0.5 kilobase) do not bind well to nitrocellulose; (2) some of the DNA noncovalently bound to the nitrocellulose is removed during posthybridization washing which subsequently reduces the intensity of the signal and limits the number of times the transfer can be reused; (3) the technique is also relatively slow (Wahl, et al., 1979).

Further discoveries led to modification of the Southern blot technique. It was shown that RNA and single-stranded DNA could be linked covalently to diazobenzyloxymethyl (DBM)-cellulose and used in hybridization reactions. RNA and small fragments of DNA could be transferred from

agarose gels to DBM paper. Transfer of DNA fragments of any size from agarose and polyacrylamide gels has now been optimized (Wahl, et al., 1979). Because the binding of the nucleic acid to the DBM paper is covalently linked, the DBM filters are reusable. This is a very real advantage when only small amounts of a particular DNA or RNA are available. Initially transferring DNA and RNA from agarose or acrylamide gels to DBM paper via blotting procedures resulted in poor transfer and relatively low covalent coupling. Stellwag and Dahlberg (1980) described an electrophoretic transfer procedure which avoided the limitations of passive blotting techniques. The electrophoretic transfer allowed efficient and rapid transfer of intact DNA, RNA, protein and ribonucleoprotein. Advantages of this technique cited by Stellwag and Dahlberg are: (1) the process is rapid allowing complete transfer within the time the paper is capable of forming covalent bonds, (2) transfer is direct, i.e., there is no lateral diffusion, preserving the sharpness of the original gel pattern and also allowing the detection of very small amounts of DNA and RNA, (3) the molecules are transferred intact, (4) the efficiency of transfer of intact RNA exceeds that by blotting and DNA transfer is equal to the method of Wahl, et al., (1979).

Stellwag and Dahlberg (1980) examined some of the parameters involved in the covalent bonding of the nucleic acid to the DBM paper. They observed: (1) that the covalent bond is formed within 2.5 hours after transfer from the gel, (2) the DBM paper's ability to form covalent bonds remains high for the first 2 to 4 hours after preparation if kept at pH 4-6 and $4^{o}C$; after this period it declines rapidly. Electrophoretic transfer from gels to paper can normally be accomplished within 2-6 hours and consequently a coupling efficiency of 60-80% can be expected.

When DNA or RNA molecules contain sequences of nucleotides that are complementary to each other, they are able to come together to form a

double helix with paired bases. When both strands are DNA the process is called renaturation or reassociation. If one strand is DNA and the other RNA, the process is called hybridization. These processes are essentially identical (Wetmur, 1976).

In order to achieve reproducible hybridization reactions the cation concentration, temperature of incubation, nucleic acid concentration and DNA fragment size all need to be controlled. Briefly the requirements are as follows: (1) There must be an adequate concentration of cations. Below 0.01M sodium ion the reassociation reaction for DNA is effectively blocked. (2) The incubation temperature of the reaction must be high enough to weaken intrastrand, secondary structure. The optimum temperature for reassociation of single strands is approximately 25°C below the temperature required for the dissociation of the double strands. (3) The incubation time and the nucleic acid concentration must be sufficient to permit an adequate number of collisions to allow annealing of the single strands. (4) The size of the genetic material also effects the rate of annealing and is conveniently controlled if the nucleic acid is sheared to small fragments (about 400-500 nucleotides long) (Britten and Kohne, 1968).

Hybridization of RNA with DNA is normally studied under reaction conditions involving elevated temperatures. The maximum rate of reaction occurs somewhere near 25°C below the mean thermal denaturation temperature (T_m) (Marmur and Doty, 1961). Exposure of nucleic acid to higher temperatures presents disadvantages such as chain scission and depurination. These difficulties may be avoided by using high concentrations of certain salts, e.g., $NaClO_4$, or by using organic solvents in which the thermal stability of double-stranded polynucleotides is greatly reduced (McConaughy, et al., 1969). This allows reactions to occur at lower incubation temperatures and often at rates of reaction several times higher than those obtained at 60 or 70°C in aqueous solution.

For hybridization of DNA:RNA to determine the degree of relationship of the base sequences, it is important not only to establish conditions for high rates of reaction but also to allow characterization of the nature of the duplexes being formed. This can be achieved by changing the temperature of reaction in order to determine the T_m of the particular heteroduplex formed. This can then be correlated with the degree of base mismatching between the two strands. Hyman, et al., (1973) estimated that for DNA:DNA heteroduplexes the T_m is reduced by 1.4°C for each percent of base mismatching between the two strands. For a heteroduplex that had 25% base mismatching (i.e., 1 in 4 bases were mismatched), the T_m would be lowered by 35°C. Hybridization reactions at high temperatures would denature heteroduplexes with a high degree of base mismatching as these would be thermally unstable. In such conditions only strands that had a high degree of relatedness would remain hybridized (high stringency conditions). At lower reaction temperatures heteroduplexes with a high degree of mismatching will be thermally stable and remain hybridized (low stringency conditions).

It can be seen that if there were a number of RNA segments that had varying degrees of relatedness when hybridized with a radiolabelled DNA probe, that the resulting heteroduplexes would have different thermal stabilities. If the probe was hybridized initially at low temperatures (low stringency) most if not all the RNA segments would remain hybridized. As the temperature of the reaction was raised, however, some of the heteroduplexes would become thermally unstable and would dissociate, till eventually at high temperatures (high stringency) only the heteroduplexes that are very closely related or identical would remain hybridized. If the temperature is measured at which a heteroduplex dissociates, the percentage of base mismatching in that duplex can be calculated from the relationship that 1 per cent mismatching lowers the T_m by 1.4°C below that of the homoduplex T_m.

As mentioned above, there are certain problems inherent with hybridizing at higher temperatures. However, the use of organic solvents can allow the same characterization of a heteroduplex as is achieved by varying the temperature of incubation. The effect of the organic solvent is to lower the thermal stability of a duplex and hence lower the temperature at which that duplex will then dissociate. If the temperature of hybridization is kept constant and the concentration of the organic solvent is increased in successive stages, the effective T_m of some of the less related heteroduplexes will be lowered to the temperature of incubation and will dissociate. If there is a known relationship between the concentration of the organic solvent and the lowering of the T_m, the actual T_m of the heteroduplex can be determined and consequently the degree of base mismatching. Organic solvents used in hybridization studies are: urea, salicylate, dimethyl sulfoxide, aromatic compounds, a number of alcohols, ethylene glycol, N,N´-dimethylformamide, and formamide (Herskovits, 1962; Geiduschek, 1962; Levine, et al., 1963).

A particularly useful organic solvent is formamide in which DNA can be denatured and renatured at room temperature (McConaughy, et al., 1969). Hybridization in aqueous formamide has distinct advantages over hybridization at elevated temperatures, including increased retention of DNA or RNA immobilized on nitrocellulose filters and decreased nonspecific background absorption. Together these two factors allow for a greater reproducibility of hybridization experiments (Bonner, et al., 1967). It has been found that 1% formamide reduces the T_m of a duplex by 0.72°C (McConaughy, et al., 1969). Consequently a duplex that would normally dissociate at 100°C, when hybridized in 50% formamide will now dissociate at 64°C.

The majority of the parameters for hybridization have been determined for DNA:DNA duplex formation. Casey and Davidson (1977) investigated the

rates of formation and thermal stabilities of RNA:DNA and DNA:DNA duplexes at varying concentrations of formamide. The important general conclusion they came to is that RNA:DNA hybrids are more stable than DNA:DNA duplexes in high formamide concentrations (90%). This is especially true for G+C rich polynucleotides. At lower formamide concentrations (0-50%) the difference in thermal stability is not as marked. In the complete absence of formamide RNA:DNA duplexes are generally slightly more stable than DNA:DNA duplexes, however as the formamide concentration is increased up to 20% the difference in stability between the two types of duplex becomes minimal and insignificant. This difference slowly increases again reaching a maximum at 90% formamide. The parameters determined by McConaughy, et al., (1969) that 1% formamide reduces a duplex T_m by 0.72°C was for DNA:DNA and DNA:RNA duplex formation.

Overall there is a lack of data on RNA:DNA duplex stability and its relationship to internal base mismatching, especially in reaction conditions using formamide. Because of this the two important parameters, that 1% base mismatching lowers the T_m by 1.4°C and that 1% formamide lower the T_m by 0.72°C, have been assumed to be true in RNA:DNA duplex formation. However, it is noted that any results determined from these 2 parameters are only estimates of strand relationship. The percentage of base mismatch in a heteroduplex can be determined from the percentage of formamide added to the reaction mix to cause denaturation at a set incubation temperature, and the T_m of the homoduplex at the same salt concentration and incubation temperature.

Heteroduplex % base mismatching

$$= \frac{T_m - (T_I + T_F)}{1.4}$$

where T_m = denaturation temperature for the homoduplex.

T_I = temperature of incubation.

T_F = Equivalent temperature effect of added formamide (1% formamide x 0.72 °C)

For example, a particular band has been lost at an incubation temperature of 52 °C. Formamide has been added to a concentration of 50% and the T_m of the homoduplex is 102 °C.

% base mismatching in the heteroduplex is at least

$$\frac{102 - (52 + (50 \times 0.72))}{1.4}$$

$$= 8.57$$

The T_m of rotaviral dsRNA has not been determined for a range of sodium saline citrate (SSC) and therefore, because rotavirus is a member of the Reoviridae, the T_m of reovirus type 3 was used as an estimate of rotavirus T_m in all calculations. Bellamy et al. (1967) determined the T_m for reovirus in different SSC - in 0.01 x, 0.1 x and 10 c SSC the RNA exhibited sharp melting profiles with T_m values of 75, 84, 96 and 104° respectively. By plotting SSC versus T_m it is possible to determine the T_m for dsRNA in the particular SSC used in the hybridization experiments. In all hybridizations of this study a 5 x SSC was used. At this concentration reovirus dsRNA has a T_m of 102 °C. This temperature was used as the estimate of the T_m for the homoduplex in all calculations of rotavirus heteroduplex mismatching.

Hybridization experiments to form RNA:DNA heteroduplexes were conducted at a constant temperature and varying concentrations of formamide. As the formamide concentration was increased certain duplexes became unstable and dissociated. The % basis mismatching in these duplexes could then be determined by the above calculation using the percentage of formamide at which the duplex dissociated. In Table 12 is summarized the relationship of heteroduplex base mismatching and formamide concentration for a number of formamide concentration. In actual experimental procedure only 0, 10 and 50% formamide was used.

TABLE 12

Relationship of Heteroduplex Base Mismatching and Formamide Concentration

Percentage Formamide	T_I (°C)	Percentage Base Mismatching
0	37	46.4
0	52	35.7
10	52	30.6
15	52	28.0
20	52	25.4
30	52	20.3
40	52	15.1
45	52	12.6
50	52	10.0

The hybridization of cDNA probes to various human and animal rotaviral isolates allowed the more precise determination of the degree of relationship hinted at by serological and electrophoretic comparisons.

2. MATERIALS AND METHODS

2.1 Preparation of dsRNA for Making Probes

Rotavirus positive (as determined by ELISA) faecal material was made into a 20% suspension with Tris Acetate buffer (0.05 M, pH 8.5). The suspension was then sonicated lightly in a sonicator bath. Ten percent lithium dodecyl sulphate (LDS) was added to an overall concentration of 1%. This was then held at 0 °C for 10 minutes with frequent mixing to allow release of virus from faecal material. The suspension was clarified by centrifugation at 9,500 g for 10 minutes and the pellet was discarded. The remaining supernatant was centrifuged at 160,000 g for 1 hour and the resulting pellet was resuspended in a small volume (less than 1 ml) of 1 x SSC and sonicated. LDS was added to 1% and lithium acetate buffer, pH 5, to 0.1 M. Viral material was then extracted with water-saturated phenol at room temperature. The aqueous phase, containing rotaviral dsRNA was re-extracted 3 to 4 times with phenol. The aqueous phase was then extracted 3 to 4 times with cold ether (4 °C) to remove any remaining phenol. The dsRNA was further purified by applying it to a 2-step $GsSO_4$-Ethidium Bromide gradient. The gradient was of $CsSO_4$ at densities of 1.6 (2.5 ml) and 1.3 (1.5 ml). The dsRNA sample (1.0 ml) was layered on top and 50 µl ethidium bromide (10 mg/ml) was added. The gradient was centrifuged overnight at 160,000 g at 4 °C. Resulting fluorescent bands were removed and dialysed against 3 changes of 0.01 M ammonium acetate, at 4 °C for 4 hours to remove the $CsSO_4$. The solution was adjusted to 0.2 M ammonium acetate prior to phenol extraction. Heated crystalline phenol (50-60 °C) in equal volume was added to the dsRNA solution. Phenol extraction was repeated, each time the volume of phenol added was halved until the volume of the aqueous phase was approximately 0.3 ml. The aqueous phase was then extracted 3 times with ether and lastly 3 volumes of ethanol (-20 °C) was added and left overnight or until required at -70 °C. Immediately prior to making a cDNA probe

the dsRNA was centrifuged at 3,000 g for 10 minutes. The pellet was resuspended in 5 mM EDTA. The purity of the dsRNA was assessed by measuring its absorbance at 260 and 280 nm. Pure dsRNA giving a 260/280 ratio of 2:1. Concentration determination was based on the assumption that an absorbance of 1 absorbance unit at 260 nm represents 40 µg per ml of RNA.

2.2 Preparation of Complementary DNA (cDNA) Probes

Preparation of ^{32}P labelled cDNA to rotaviral dsRNA was by the method of Gould and Symons (1977) and Taylor et al. (1976). The reaction mixture of 125 µl contained 3.4 µmoles Tris-HCl pH 8.3; 0.6 µmoles dithioerythritol; 0.23 µmoles $MgCl_2$; 7.6 µmoles KCl; 0.03 µmoles dCTP, dGTP and dTTP; 0.6 nmoles dATP; α-^{32}P-dATP 5-50 µc, denatured rotaviral RNA 12-30 µg, DNAse I digest of calf thymus DNA as primer 30 µg; and 12.5 units avian myeloblastosis virus reverse transcriptase.

Rotavirus RNA was boiled for 3 minutes then rapidly cooled on ice immediately prior to addition to the above reaction mix. The reverse transcriptase was added after the addition of the RNA template. The reaction mix was incubated at 42 °C for 90 minutes. Synthesis of CDNA was stopped by the addition of sodium dodecyl sulphate to 0.1%. Sodium hydroxide was added to 0.5 M and incubated for either 1-2 hours at 42 °C or overnight at room temperature to hydrolyze the RNA template. The NaOH was neutralized with 1/10 total volume of 1 M Tris acetate, pH 7.5, and 1/20 volume 11M HCl. The cDNA was freed from unincorporated radioactive nucleoside triphosphates and low molecular weight material by passage through a 3.0 mm x 200 mm sephadex G-100 column. The rotavirus cDNA was collected in fractions and pooled. This was then used directly for hybridization studies.

2.3 Estimation of Efficiency of cDNA Synthesis

Small volume samples were taken at various stages to allow determination of efficiency of incorporation of the radioactive nucleoside

triphosphates into the cDNA produced. A 2µl sample was taken at time 0 (i.e., immediately after the addition of the reverse transcriptase) and at time 90 minutes, after termination of the reaction. To determine the amount of label in the cDNA, these samples were precipitated in 1ml of trichloroacetic acid (TCA) to which bovine serum albumin (BSA) was added, as carrier, to a final concentration of 100µg per ml. The samples were then filtered onto glass fibre filters. A third sample of 2µl, taken at time 90 minutes was dried directly onto a glass fibre filter to determine the total amount of ^{32}P in the reaction mix. The filters were dried and the radioactivity counted.

The cDNA and free triphosphates were separated on the sephadex column. Each fraction collected was counted for radioactivity to determine where the two peaks were. These counts were in Cerenkov units which are converted to toluene counts on the basis that Cerenkov counts are approximately 40% as efficient as toluene.

The total number of CPM recovered in cDNA compared with the number of counts added to the reaction mix initially was determined as a percentage and taken as an indication of the efficiency of incorporation of ^{32}P uptake into cDNA and hence of cDNA production from the RNA template.

2.4 Electrophoretic Transfer of Viral RNA to Diazobenzyloxymethyl (DBM) Paper

Essentially the methods of Stellwag and Dahlberg (1980) were used. Before transfer to DBM paper, the RNA that had been electrophoresed on polyacrylamide gels was trimmed to size to remove excess gel. The gel was then soaked in 0.1M sodium hydroxide for 15 minutes at room temperature to cleave the RNA and reduce the average molecular size in order to improve elution. The alkali soaked gel was then transferred to successive changes of 500mM and 25mM sodium phosphate (pH 5.5) for 15, 5 and 5 minutes respectively at 0°C.

DBM paper, frozen at -70°C was freshly thawed during the gel washes above and washed successively, 5 times, in cold distilled H_2O, twice in 0.2M sodium acetate, pH 4.0 and briefly in 25mM sodium phosphate, pH 5.5. The gel was then transferred into an electrophoretic transfer cell. Electrophoretic transfer of the dsRNA from the gel to the DBM paper was carried out in 25mM sodium phosphate buffer, pH 5.5 at 0°C, 10 volts per cm and 2.5mA for 4 hours. At the end of the transfer period the DBM was separated from the gel, rinsed in 0.1% SDS, blotted dry and stored at -70°C prior to hybridization with cDNA. The gel was discarded as very little dsRNA would be retained.

2.5 Preparation of RNA for cDNA Synthesis from a Single SA_{11} RNA Segment

For resolution of all genome segments and to lower contamination when removing a single band from a polyacrylamide gel, 5% polyacrylamide gels were prepared using a 90mM Tris-borate, pH 8, 2.5mM EDTA buffer system. Rotavirus RNA segments (5-8µg) were resolved after electrophoresis at 600V and 22mA for 8 hours. Bands were visualised by staining with ethidium bromide (50µg per ml) and were excised into siliconized plastic tubes previously packed with siliconized glass wool. The gel containing the single band was broken up and the RNA eluted out overnight at 37°C in elution buffer - 0.5M ammonium acetate; 0.01M magnesium acetate; 0.1mM EDTA; 0.1% SDS. The bottom of the tubes was pierced and the elution buffer expelled from the tube by low speed centrifugation. The tube was rinsed with elution buffer, spun and the two fractions of elution buffer pooled. RNA was ethanol precipitated on dry ice for 20 minutes, pelleted by low speed centrifugation and resuspended in 10µl of 5mM EDTA. This preparation could then be used for probe production.

A later modification to further purify the RNA from contaminating acrylamide and salts was to phenol extract the RNA with 50µl of water

saturated phenol at room temperature. The phenol phase was discarded and any remaining phenol removed by ether extraction. The RNA was again precipitated by ethanol on dry ice for 20 minutes. The resulting precipitate was pelleted and resuspended in 10µl of 5mM EDTA. This preparation was then used directly for probe production. Probes were made by the same procedure as for the whole rotavirus genome except scaled down in volume so that the final volume was only 20-30µl.

2.6 Molecular Hybridization

The methods used were essentially those of Alwine, et al., (1979) and Wahl, et al., (1979). After the transfer of RNA to the DBM paper it was blocked with a prehybridization mix to remove any remaining diazonium groups and to add carrier compounds. Prehybridization mix contained 50% formamide, 0.1% (w/v) each of BSA, ficoll and polyvinyl pyrrolidone, 1% (w/v) glycine, 50mM sodium phosphate, pH 6.5; 0.075M sodium citrate, 0.37M sodium chloride, 25µg per ml of sonicated denatured calf thymus DNA. Prehybridization reactions were carried out in sealed plastic bags in a 52°C water bath for one hour, a volume of 3.5 ml of prehybridization mix was used per bag.

For hybridization, cDNA (approximately 10^6 CPM) was boiled for 3 minutes, rapidly cooled on ice and added to hybridization mix, preheated to 52°C. This was mixed and added immediately to the DBM paper and allowed to hybridize with the RNA for 6 hours at 52°C. Hybridization mix differed from prehybridization mix in having a lower calf thymus DNA content - 10µg per ml, no glycine, dextran sulphate to 10% and varying concentrations of formamide from 0-50% according to the conditions desired (see Table 12).

After incubation, the DBM paper was washed for 15 minutes, two times with 250ml of 0.36M NaCl; 20mM sodium phosphate, pH 7.0; 2mM EDTA; 0.1% SDS at room temperature, followed by two 250ml washes with 18mM NaCl; 1mM sodium phosphate, pH 7.0; 0.01M EDTA; 0.1% SDS for 15 minutes each at 37°C.

The DBM paper was then blotted dry, sealed in a plastic bag and autoradiographed at -70°C using Kodak X-Omat-S X-ray film with a Dupont Cronex Lighting Plus intensifying screen. Several exposures were made with each hybridization usually for 18-40 hours.

Hybridized cDNA could be lifted from the DBM paper without removing the covalently bound RNA by incubating the paper with 95% formamide; 0.02M Tris-acetate, pH 7.5; 1mM EDTA in a sealed plastic bag at 75°C for 1 hour. The paper was then washed in 250ml of 0.36M NaCl; 20mM sodium phosphate, pH 7.0; 2mM EDTA; 0.1% SDS at 75°C. The DBM paper with bound RNA could then be reprobed with fresh cDNA to a different rotavirus isolate or else stored at -70°C.

3. RESULTS

3.1 Electrophoretic Transfer of dsRNA from Slab Gel to DBM Paper

To determine whether all dsRNA bands were being transferred in equivalent amounts, ^{32}P-labelled reovirus-type 3, dsRNA was run in 17 channels at a concentration of 0.5µg per channel, by PAGE at 150V for 8 hours. Double-stranded RNA bands were stained with ethidium bromide (5µg/ml) and visualized with UV light. Of the 10 dsRNA segments, 9 were resolved with segments four and five migrating together. The gel was trimmed of any excess gel not containing RNA, digested in 0.1M NaOH and washed in 500mM, 50mM and 25mM sodium phosphate buffer and the RNA electrophoretically transferred to freshly prepared DBM paper. After rinsing in 0.1% SDS, the DBM paper was exposed to X-ray film overnight at -70^{o}C. The gel was also exposed to X-ray film under identical conditions to determine the amount of radioactive dsRNA was remaining in the gel.

Results in Figure 21 show that all the bands are electrophoretically transferred from the gel to the DBM paper. There appears to be areas of slightly increased efficiency of transfer, particularly at the edges of the gel.

3.2 Synthesis of cDNA Probes

The incorporation of deoxyadenosine 5'-[x-^{32}P] triphosphate in cDNA using denatured rotaviral dsRNA as template varied in efficiency. cDNA probes were made from SA_{11} rotavirus RNA template (x 4); NCDV (x 4); canine rotavirus-CN/DUN/59/79 (x 2) and bovine rotavirus-BO/DUN/77/79.

The general pattern was for a low level of ^{32}P-ATP incorporation into cDNA averaging 11.7% for the 11 probes synthesized. There were two exceptions, however, -SA_{11}-a) and canine-a) both showed relatively high efficiency in cDNA synthesis -22 and 45% respectively (see Table 13).

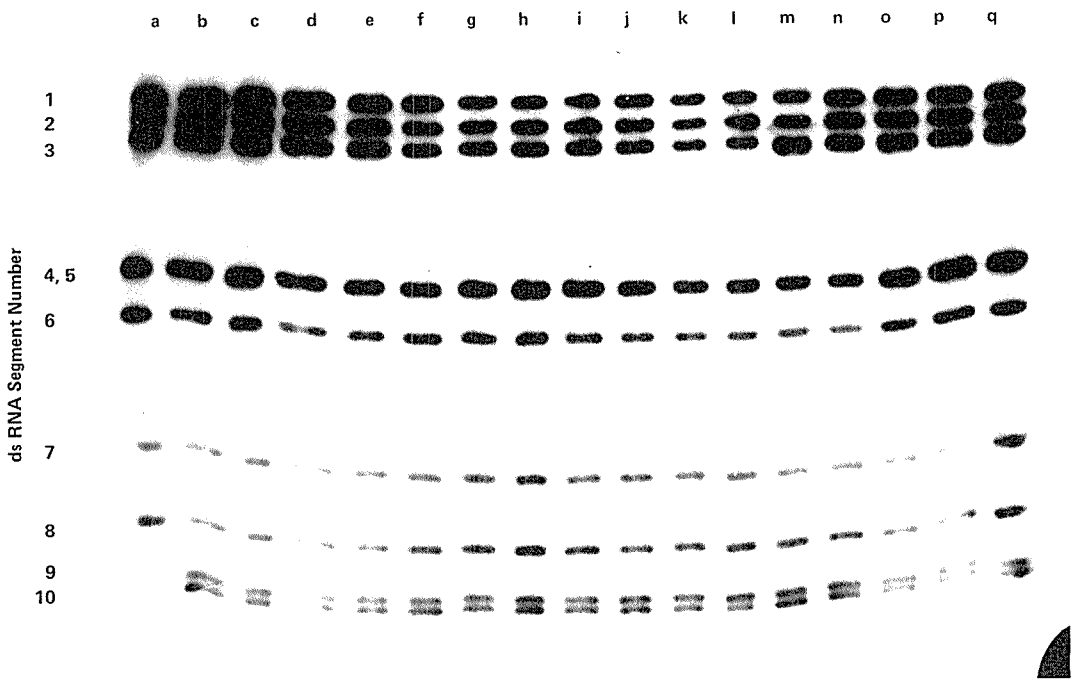

Fig. 21--dsRNA (^{32}P) Electrophoretic Transfer from Polyacrylamide Gel to DBM Paper. Reovirus dsRNA (^{32}P) 0.5µg per channel was electrophoretically transferred from a polyacrylamide gel to DBM paper at 10 volts per cm and 2.5mA for 4 hours at $0^{o}C$ in 25mM sodium phosphate buffer. The DBM paper was exposed to X-ray film at $-70^{o}C$ for 18 hours.

TABLE 13

cDNA Probe Synthesis

RNA Template	Percentage of ^{32}[P] ATP* Incorporated into cDNA
SA$_{11}$	
a	22
b	6.6
c	7.8
d	1.3
NCDV	
a	10.8
b	3.1
c	8.1
d	4.4
CN/DUN/59/79	
a	45
b	9.3
BO/DUN/77/79	
a	10.3

* Calculated from TCA precipitations of the reaction mix after 90 minutes (see 2.3)

After cDNA synthesis, the cDNA was separated from free triphosphates by passing through a sephadex column. The total amount of ^{32}P label recovered at the bottom of the column was always much less than the amount added to the original reaction mix. From the 11 probes made, an average of only 16% of the $^{32}[P]$ ATP added in the initial reaction was recovered from the column. Approximately 84% of the $^{32}[P]$ ATP was being lost at some stage during the reaction and separation steps. In the initial step, the triphosphates may have been binding to the walls of the plastic tube, this could have been avoided by using siliconized tubes. After probe and free triphosphate separation the sephadex column retained a high level of radioactivity which could have accounted for a large amount of the lost radiolabel.

Although satisfactory and usable amounts of cDNA probe were synthesized, it would have been advantageous to produce probes with higher levels of radioactivity and thus be able to have shortened the exposure times needed for autoradiography.

3.3 Synthesis of cDNA to a Single Segment of SA_{11} RNA

Initial preparations of cDNA probes to single segments of SA_{11} rotaviral dsRNA yielded very little cDNA. The average incorporation of $^{32}[P]$ ATP for 3 probes was only 0.04%. Even when the total amount of probe made was hybridized with rotaviral RNA there was not enough activity to autoradiograph within a reasonable time period. These initial probes were made using template RNA only purified as far as the first ethanol precipitation in the preparation procedure (see 2.5). At this stage acrylamide and salts as contaminants are probably still in quite high concentrations and it was considered that these may inhibit the reverse transcriptase. To check that it was a problem of inhibition of cDNA production rather than a loss of RNA template during its purification, $^{32}[P]$ labelled reovirus RNA was treated in the same way as SA_{11} RNA for purification of a single segment.

Reovirus RNA (10μg) was electrophoresed on a 5% polyacrylamide gel using the TBE buffer system at 600V, 22mA for 10 hours. Bands were stained with ethidium bromide and single bands were cut out and ground in siliconized plastic tubes packed with siliconized glass wool. The RNA was left to elute into the buffer overnight. The eluate was spun out of the tube and RNA precipitated with ethanol on dry ice. The eluate, glass wool, tube and ethanol were all checked for radioactivity and compared to the initial level of radioactivity in the single band. For 2 bands that were treated in this way, the recovery of RNA after ethanol precipitation was 71 and 66 percent. Most of the RNA was lost in the glass wool (13%) with the rest being lost during manipulation steps and in the ethanol. An equivalent amount of gel, without any RNA, was treated in like manner throughout as a control.

A single band of reovirus RNA contained approximately 1.0μg of RNA (1/10 of the original amount as there are 10 segments), therefore a 70% recovery of RNA would mean approximately 0.7μg of RNA was recovered. To test the efficiency of this preparation of RNA as template for cDNA synthesis an assay was set up with four variables:

1) reovirus RNA (0.74μg) from band 6 purified as above;

2) reovirus RNA (0.97μg) purified;

3) reovirus RNA (0.97μg) purified + acrylamide material from control above to 10%;

4) reovirus RNA (0.97μg) purified + acrylamide material from control above to 90%.

Step 1) tested ability to synthesisecDNA from RNA eluted out of an acrylamide gel, 2) was to show the level of cDNA synthesis from pure RNA that had no acrylamide material present, 3) and 4) were to show the effect of adding acrylamide to purified RNA to see if inhibition occurred and whether this increased with more acrylamide material added. The efficiency of cDNA synthesis for each preparation of RNA template is summarized in Table 14. Addition of acrylamide material to pure reovirus RNA at 10%,

TABLE 14

Effect of Contaminating Acrylamide on cDNA Synthesis

RNA Template	Total CPM Added	cDNA CPM Recovered	% Recovery of ^{32}P in cDNA
Single Band RNA (0.7 g)	2.1×10^6	252	0.01
Purified RNA (0.9)	2×10^6	5.3×10^5	26.5
Purified RNA (0.9 g) + 10% contaminant	2×10^6	2.7×10^5	13.9
Purified RNA (0.9 g) + 90% contaminant	1×10^6	366	0.04

decreased cDNA production by half, 90% acrylamide material virtually brought cDNA synthesis to the level of that for the single band template - 1.) From these results it was concluded that contaminating material still present after precipitation of the single band RNA in ethanol was inhibiting cDNA production.

In an attempt to further purify the RNA template the procedure was modified by including a phenol and ether extraction with a further ethanol precipitation (see 2.5).

Band 2 of SA_{11} rotavirus RNA was purified by the modified procedure and used as template for cDNA production. Efficiency of ^{32}[P] ATP incorporation was 55 times greater than for previous attempts but was still only 2.2% of the ^{32}[P] ATP added to the reaction mix initially. However, there was adequate radioactive probe in this preparation to hybridize to RNA and get a result by autoradiography within 7 days.

3.4 Hybridization of SA_{11} Rotavirus cDNA Probe

3.4.1 To Cultivable Rotaviruses - NCDV, NI, WA and SA_{11}. Radioactively labelled (^{32}[P]) cDNA to SA_{11} RNA was hybridized against the RNA of four cultivable rotaviruses: SA_{11}, NCDV, NI and WA. The stringency of hybridization was varied by addition of formamide at different concentrations to the hybridization mix. Probes were hybridized over a range of 0-50% formamide (Fig. 22).

At 0% formamide the SA_{11}-cDNA probe hybridized with all bands of the homologus SA_{11} RNA indicating that cDNA had been synthesized from all 11 segments. When hybridized against NCDV, NI and WA RNA all bands had hybridized except band 5; band 4 was also absent with WA RNA. The conditions of hybridization were made less stringent by keeping the formamide at 0% and lowering the temperature of incubation from 52°C to 37°C (Fig. 24). Under these conditions band 5 of NCDV had hybridized. At this stringency

Fig. 22--Hybridization of SA_{11} cDNA to Cultivable Rotavirus dsRNA.

a) 0% formamide.

b) 10% formamide.

c) 30% formamide.

d) 50% formamide.

Autoradiography was for 18 hours at -70°C.

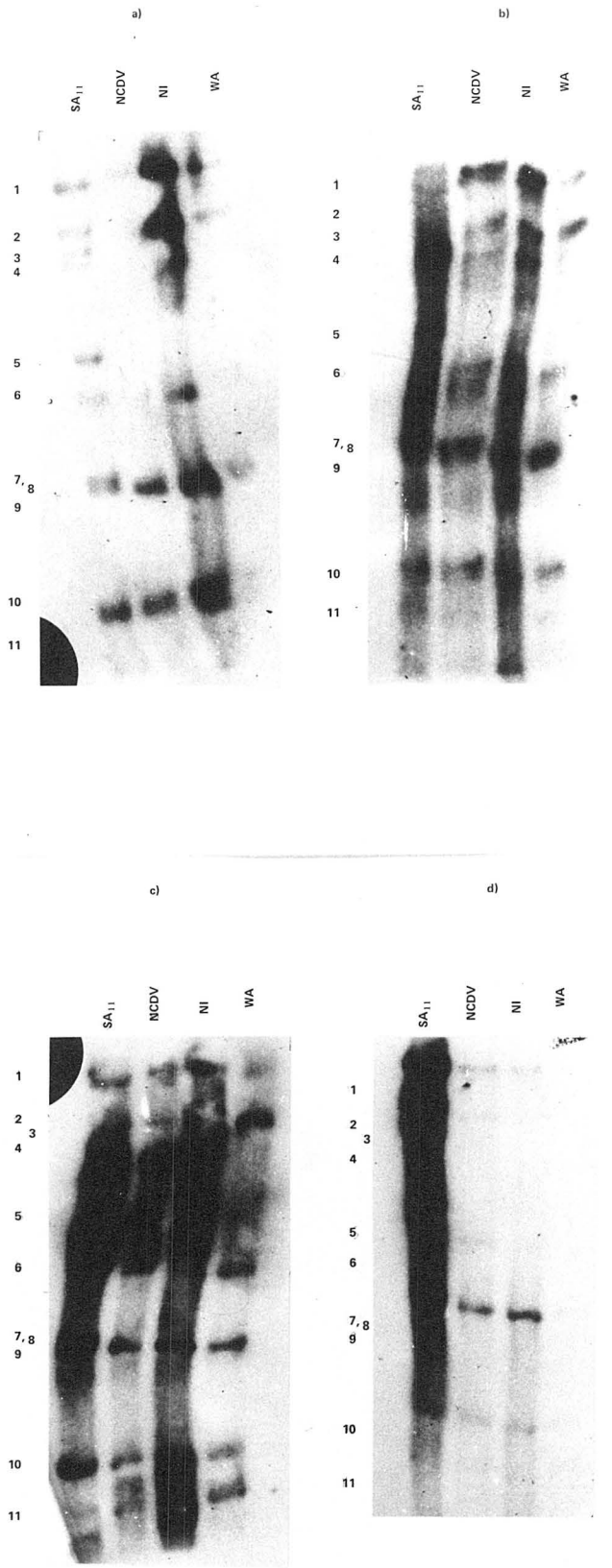

Fig. 22

cDNA-RNA duplexes will stabily bind with up to 46% of the genetic material being mismatched. This faint band was lost when the stringency was increased to 0% formamide but 52°C incubation (i.e., a base mismatch equivalent of 35.7%). Bands 4 and 5 of WA virus were absent at 0% formamide and 52°C indicating that there is greater than 35.7% base mismatching between SA_{11} and WA in these segments. At this stringency band 4 was present for both NCDV and NI, and at 30% formamide, 52°C, but was lost when the stringency of hybridization was increased to 50% formamide at 52°C, an equivalent base mismatching stability limit of 10%. This indicates that segments 4 and 5 of SA_{11}, NCDV and NI have somewhere between 20.3 and 10% mismatch. At 50% formamide and 52°C NCDV and NI showed evidence of hybridization in bands 1-3 and 6-10 with the band containing segments 7, 8 and 9 showing very strong hybridization. At this stringency WA virus showed very little hybridization with SA_{11} cDNA.

Of the cultivable series then it appears that the calf rotaviruses NCDV and NI are more closely related to SA_{11} than is the human WA rotavirus.

3.4.2 SA_{11} Hybridization to Human and Animal Rotaviral RNA. SA_{11} cDNA probes were hybridized against a number of animal and human rotaviruses at three different stringencies. Firstly, at 0% formamide, 37°C which allows stable hybridization of DNA:RNA with up to 46.4% base mismatching. Secondly, at 0% formamide and 52°C, allowing for up to 35.7% base mismatching, and thirdly, 50% formamide and 52°C, allowing for up to 10.0% base mismatching.

At 0% formamide and 37°C segment 5 showed no hybridization with the calf and human rotaviruses but some faint hybridization with the dog and foal isolates. Band 3 and 4 were only just visible for the foal isolate. All other segments showed hybridization under these conditions (Fig. 24).

When the stringency was increased to 0% formamide and 52°C the human isolates lost hybridization to segment 4. It can be concluded that this segment has base homology between SA_{11} and human isolates of at least 53.6%

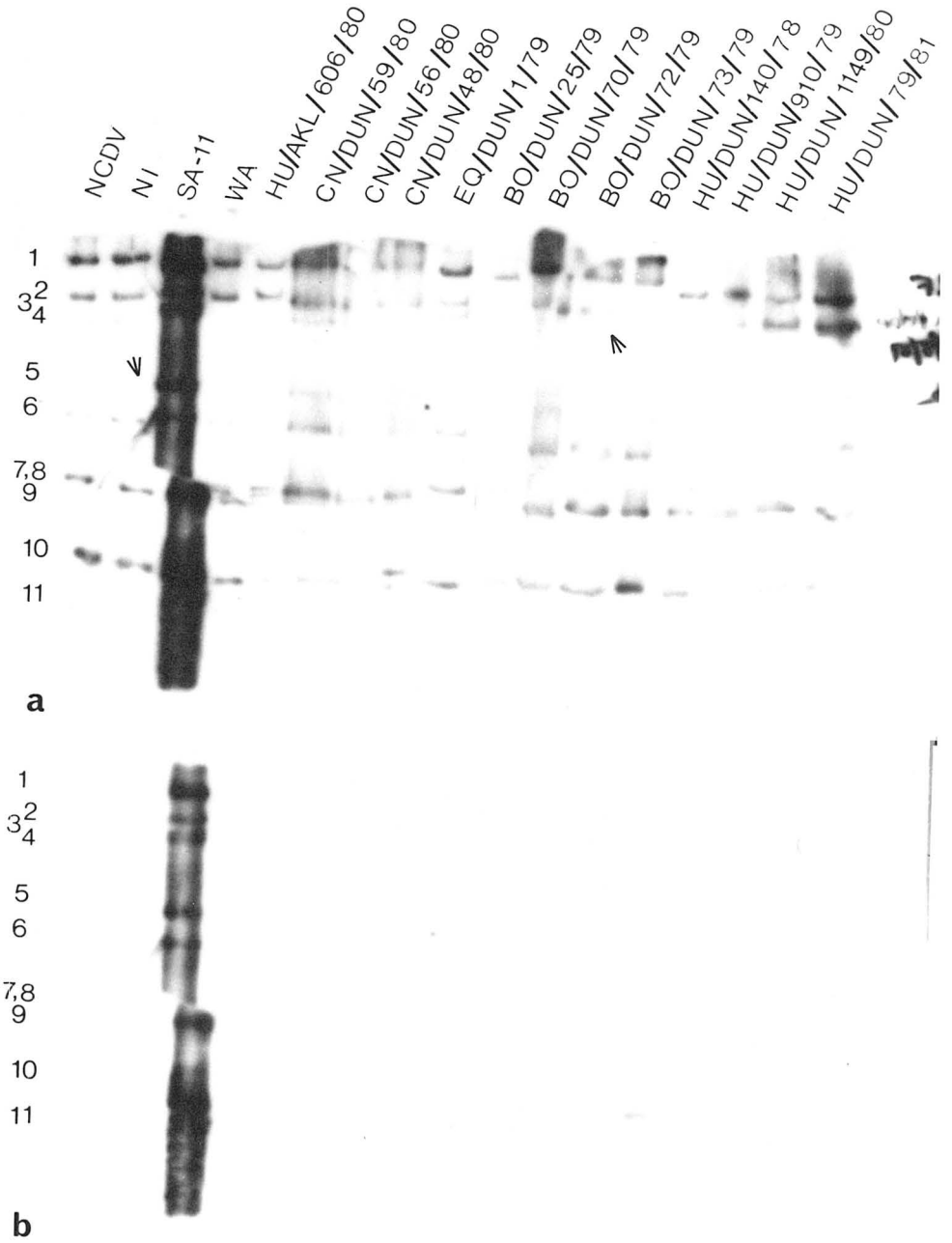

Fig. 23--Hybridization of SA_{11} cDNA to Human and Animal Rotaviral dsRNA.

a) Hybridization at 0% formamide.

b) Hybridization at 50% formamide.

Arrows show absence of bands. Autoradiography was for (a) 66 hours, and (b) 112 hours.

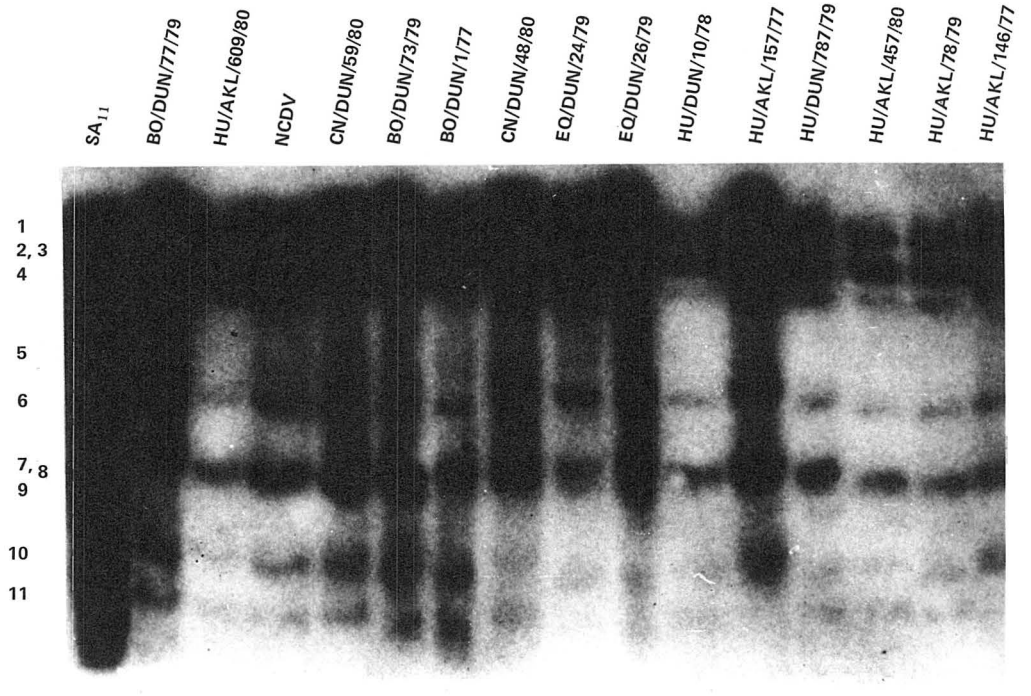

Fig. 24--Hybridization of SA_{11} cDNA to Human and Animal Rotaviral dsRNA at very Low Stringency. Hybridization was at 0% formamide and 37°C allowing for stable binding with up to 46% of the genetic material mismatched.

but no greater than 64.3%. At these more stringent conditions no other bands were lost with the other isolates (Fig. 23).

Under high stringency conditions (50% formamide and 52°C) where only closely related (at least 90%) sequences will stabily hybridize, all isolates, human, and animal, lost the majority of the remaining segments, only bands 7-10 showed faint hybridization.

Because there was very little hybridization under high stringency conditions it appears that SA_{11} rotavirus is not closely related to any of the locally isolated calf, human, dog or foal rotaviruses. However, there is some relationship at least up to 53.6% of the base sequences in 9 of the 11 segments. How much greater the relationship could only be determined by hybridizations at stringencies between 0% and 50% formamide at 52°C to determine at what point the heteroduplexes were becoming thermally unstable.

3.5 Hybridization of NCDV cDNA Probes

3.5.1 To Cultivable Rotaviruses - NCDV, NI, WA and SA_{11}.

NCDV cDNA probes were hybridized against cultivable rotavirus RNA at two different stringencies. Firstly, at 0% formamide and 52°C which allows for hybridization of cDNA:RNA with up to 35.7% base mismatching, and secondly, at 50% formamide and 52°C allowing for up to 10% mismatch.

At low stringency (0% formamide, 52°C) in the homologous hybridization of NCDV cDNA to NCDV RNA there was hybridization with all bands. Band 11, however, was faint indicating that there may be an inefficient synthesis of cDNA to this band. NCDV cDNA hybridized to all the bands of NI, all bands except 5 with SA_{11}, and all bands except 4 and 5 with WA rotavirus (Fig. 25, channels 1-4).

At increased stringency (50% formamide and 52°C) NI RNA still showed complete hybridization with NCDV cDNA. SA_{11} showed relatively strong

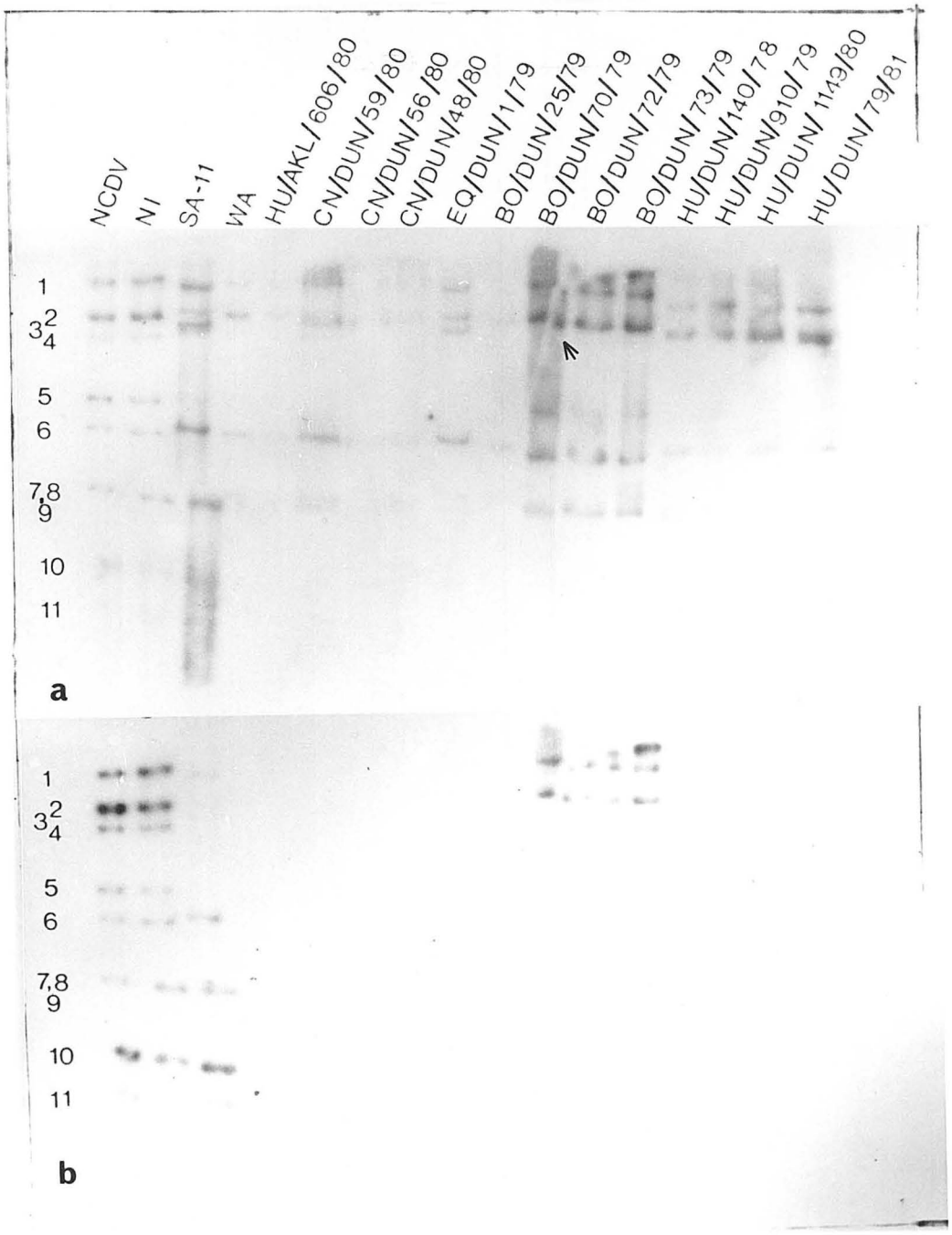

Fig. 25--Hybridization of NCDV cDNA to Rotaviral dsRNA.

a) Hybridization at 0% formamide.

b) Hybridization at 50% formamide.

Arrow indicates absence of band 4 on bovine isolates. Autoradiography was for (a) 72 hours, and (b) 64 hours.

hybridization with bands 6 to 11 and WA showed only faint hybridization with band 10.

From these results it can be concluded that NCDV and NI rotaviruses are very closely related, at least 90% of their base sequences being identical. Even at high stringency the intensity of the NI hybridization was as strong as with the homologous NCDV vs NCDV situation.

NCDV and SA_{11} rotaviruses again showed a relatively high degree of similarity in bands 6 to 11. The human virus, WA, is genetically quite different to NCDV with hybridization only occurring at low stringency conditions that allow for a large amount of dissimilarity (35.7%).

3.5.2 NCDV Hybridization to Human and Animal Rotaviral RNA. NCDV cDNA was hybridized with calf, dog, foal and human rotaviral RNA at low (0% formamide, 52°C) and high (50% formamide, 52°C) stringencies.

At low stringency the NCDV probe hybridized with all bands except 4 and 5 of dog; 4, 5 and 11 of human; 5, 7 to 11 of foal; 4 and 11 of calf rotaviruses. It is notable that only foal rotavirus RNA showed hybridization with NCDV segment 4 (Fig. 25).

At high stringency only the local calf rotaviral RNA showed strongly hybridized bands (1-3, 6-9). There was faint hybridization with bands 5 and 10 of calf isolates; 7 to 11 of dog; 2 and 10 of foal; and 2, 3, 7 to 10 of human rotavirus RNA. (Fig. 25).

The calf rotavirus isolates are the most closely related to the NCDV cDNA probe with segments 1-3 and 6-9 hybridizing as strongly under high stringency as did the homologous NCDV cDNA:NCDV RNA. Bands 5, 10 and 11 showed only weak hybridization and therefore showed some similarity but band 4 was quite different with no hybridization even at low stringency. Band 4 then is at least 35.7% different in genetic sequence between NCDV and local calf rotavirus isolates. Of the other rotavirus isolates, none are closely related in any of their segments to NCDV.

3.6 Hybridization of Dog cDNA Probes

3.6.1 To Cultivable Rotaviruses - NCDV, NI, WA and SA_{11}. Dog cDNA was hybridized with NCDV, NI, WA and SA_{11} rotaviral RNA at low stringency (10% formamide and 52°C, allowing for up to 30.6% base mismatching) and high stringency (50% formamide and 52°C) (Fig. 26, channels 1-4). Production of cDNA to bands 10 and 11 appeared to be deficient as in the homologous hybridization of dog cDNA to dog RNA there was only weak hybridization compared with the other bands when autoradiographed. Consequently, it is hard to evaluate the absence of bands 10 and 11 in the heterologous hybridizations. All other bands were synthesized during cDNA production.

At low stringency band 5 is absent from all the isolates. Band 4 shows homology with all 4 virus isolates but is faint in WA virus. Bands 1-3, 6-9 all show strong hybridization when compared with the homologous hybridization.

When the stringency for hybridization was increased bands are largely removed in all four isolates. There is faint hybridization however with bands 1-3 and 7-9 in all 4 viruses. All bands are still strongly hybridized in the homologous situation at high stringency.

From these results it appears that dog rotaviral cDNA is not closely related to any of the cultivable rotaviral RNA genomes.

3.6.2 Dog Rotavirus Hybridization to Human and Animal Rotaviral RNA. Inefficient copying of segments 10 and 11 in the synthesis of dog rotaviral cDNA meant that conclusions relating the degree of homology of dog rotavirus cDNA and other isolates RNA in segments 10 and 11 could not be made. Hybridizations were done at low (10% formamide, 52°C) and high (50% formamide, 52°C) stringency (Fig. 26).

At low stringency, dog cDNA hybridized with bands 1, 2 and 3 of human and foal rotavirus but only weakly with calf. Human, foal and calf all failed to hybridize in band 4. Band 5 of foal rotavirus did hybridize but

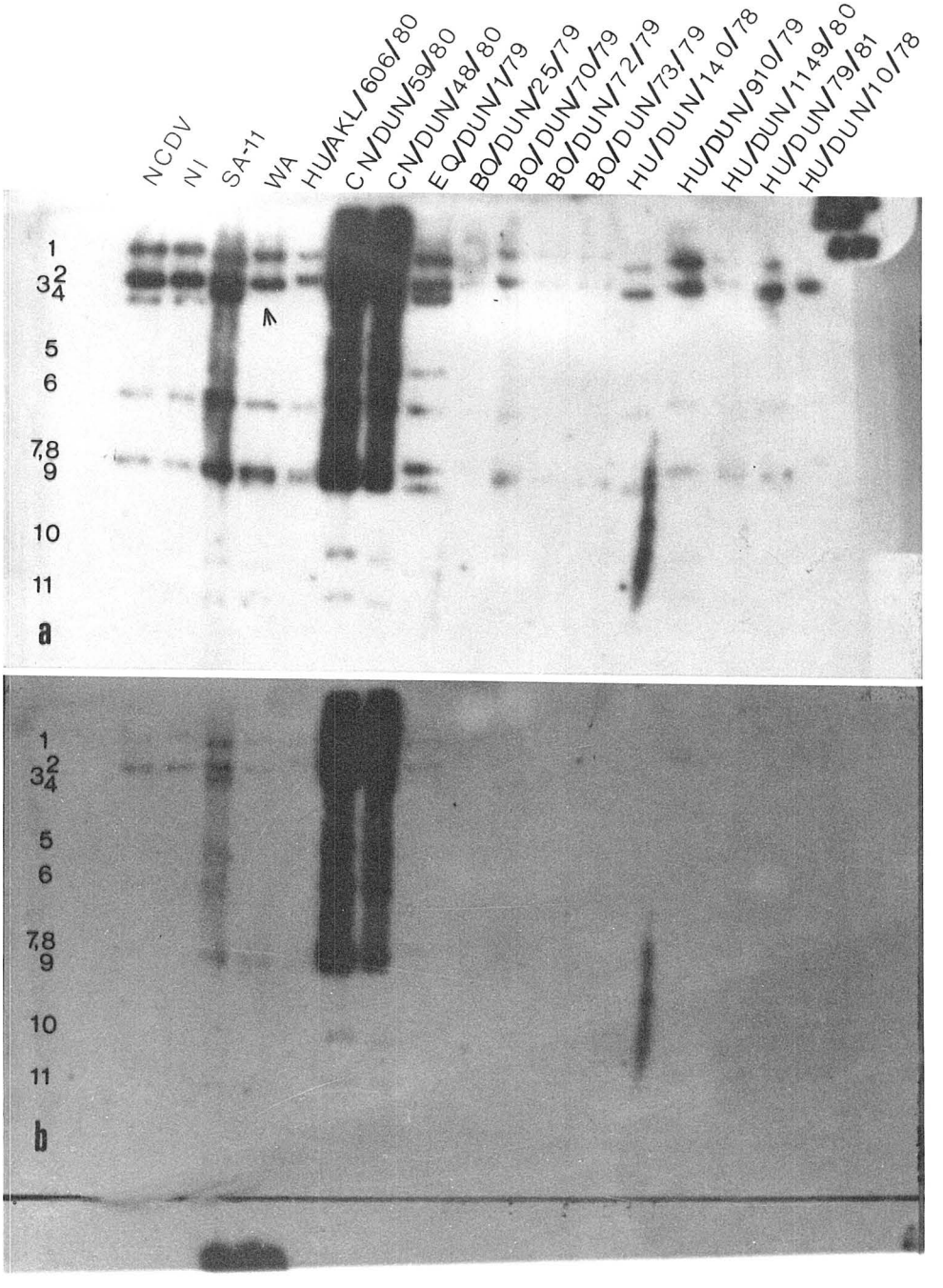

Fig. 26--Hybridization of Dog Rotavirus cDNA to Rotaviral dsRNA.

a) Hybridization at 10% formamide, 52°C (arrow indicates absence of band 4 on WA rotavirus).

b) Hybridization at 50% formamide, 52°C.

this band was absent in other isolates. Band 6 hybridized with foal and partially with some human isolates, but was absent with calf. Bands 7 to 9 of foal rotavirus hybridized with [illegible] only partial hybridization to human isolates; calf isolates showed no hybridization.

When the stringency of the hybridization was increased hybridization to human and calf rotaviral isolates ceased and only weak hybridization in bands 1, 2 and 7, 8 was evident in foal rotavirus.

From these results it appears that the dog rotavirus cDNA is distinctly different in genetic sequence to all other animal and human rotavirus isolates. At low stringency the dog and foal rotaviruses appear to be related but any relationship is removed under high stringency conditions. Bands 4 and 5 again appear to be the most dissimilar between the various isolates.

3.7 Single-Band Hybridization

This hybridization was designed to show that hybridization of cDNA and RNA of different rotaviral isolates is occurring specifically between equivalent segments and was not just a random event. A cDNA probe to one RNA segment, SA_{11} segment 2, was produced and hybridized with the full genomes of NCDV, NI, WA and SA_{11} rotaviruses at low stringency. If the hybridization that has occurred in previous experiments was not specific then in this hybridization a number of bands would be expected to be radioactive, however, if the hybridization is specific then only segment 2 should hybridize for all four isolates.

Upon autoradiography it was evident that the cDNA probe had hybridized strongly with band 2 of all 4 isolates and partially with band 1 of SA_{11} RNA (Fig. 27).

The partial hybridization of cDNA of segment 2 with band 1 could be explained by some retention of segment 2 molecules with the larger, slower migrating molecules of segment 1 in the PAGE.

From this experiment segment 2 probe is binding to the equivalent band in other rotaviral isolates. It is too much to deduce from this result that all segments are hybridizing only with their equivalent segment in heterologous hybridizations but this is likely to be the case. Segments that would be particularly interesting to probe individually are 10 and 11 which appear to be inverted in some human isolates.

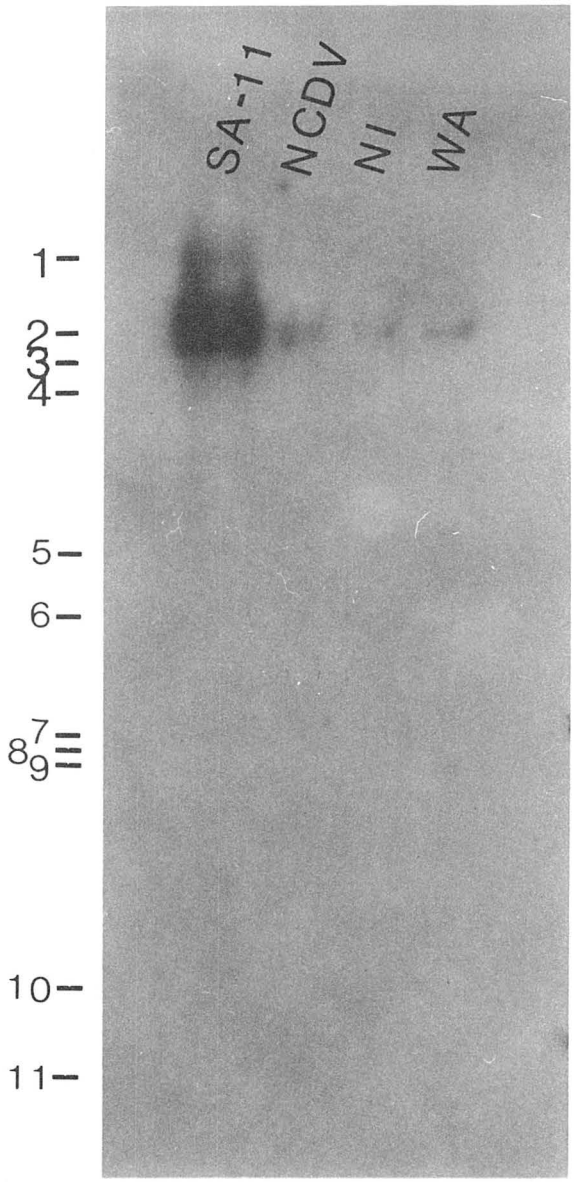

Fig. 27--SA_{11} Rotavirus RNA Segment 2 cDNA Probe Hybridized with Cultivable Rotavirus dsRNA. Hybridization was at 0% formamide and 52°C.

4. DISCUSSION

The results of hybridization experimentation indicate that all the rotavirus isolates, cultivable and noncultivable are related to the extent that hybridization occurs at low stringency. However, they are not that closely related that stable hybrids form at high stringency. The two exceptions were the two cultivable bovine isolates NCDV and NI. Double-stranded RNA from these two isolates remained hybridized under the most stringent of conditions. In these conditions base mismatching only up to 10% of the genetic sequence would be tolerated. Hence, the NCDV and NI isolates are either identical or at least very closely related. This is of particular interest considering the two isolates came from widely separated parts of the world. Of the other cultivable viruses the human WA virus is quite distinct from the two bovine and the SA_{11} rotaviruses. SA_{11} does show some relationship with the bovine viruses at high stringency in all segments except 4, 5, 6 and 11.

Of the probes hybridized against noncultivable, local rotavirus isolates, only NCDV showed any significant relationship under high stringency. NCDV and the local calf rotavirus isolates hybridized to a level of 90% homology in segments 1-3, 6 and in the band containing segments 7, 8 and 9. Segment 10 showed evidence of hybridization at high stringency but of a lower level than the other segments.

It is not fully understood why at high stringencies some bands are faintly seen by autoradiography. One possible explanation is that during the synthesis of the cDNA probe there are some very short pieces of DNA made that are homologous with a section of the RNA and so hybridize stabily but that these short pieces are in low concentration and so do not produce high levels of radioactivity.

Of all the segments, 4 and 5 show the least homology between all the isolates. NCDV probe, hybridized against the local calf isolates, showed a high degree of homology in most segments but still showed very little homology in segment 4. Segments 5, 10 and 11 all showed minor homology at high stringency.

Unfortunately segments 7, 8 and 9 often migrate as one or two bands and consequently it is not possible with most isolates to distinguish whether all three segments have hybridized or if 1 or 2 are missing. With some of the isolates it appears that the 9th segment may not be hybridizing.

A number of attempts have been made to assign proteins and functions to particular gene segments. There is some disagreement as to the coding assignments of these segments and there is no universal nomenclature for the protein products. However, some general points can be made. Rotaviral RNA segments 1, 2 and 3 all code for inner proteins I_1, I_2 and I_3, respectively. Segment 5 codes for an outer shell protein O_1, thought to be the haemagglutinin antigen (Matsuno, et al., 1980) and along with the product of segment 4 is thought to play an important role in cell tropism and perhaps host range (Kalica, et al., 1981). The sixth segment codes for the major inner shell polypeptide I_4 (Smith, et al., 1980) which is thought to determine rotavirus subgroups as determined by the immune adherence haemagglutination assay (Kalica, et al., 1981). Segments 7, 8 and 9 code for three polypeptides, one being a protein of the outer shell, O_2, and two nonstructural proteins, NS_1 and NS_2. Greenberg, et al., (1981), based upon their analysis of reassortants of human and bovine rotaviruses, suggested that segment nine was a likely candidate to code for the protein with which neutralizing antibodies interact. Kalica, et al., (1981), with further studies of human and bovine rotavirus dsRNA reassortants concluded that the ninth RNA segment codes for an outer protein that induces and reacts/neutralizing antibodies. Segments 10 and 11 may alternate in order with

depending on whether the electrophoretic profile is the "long" or "short" pattern. For viruses with the "long" pattern, segments 10 and 11 may code for a nonstructural protein NS_3 or an outer shell protein O_3, and an outer shell protein O_4, respectively. The O_4 protein is known to be glycosylated (Dyall-Smith, et al., 1981) and is thought to be a major determinant of ELISA specificity of rotaviruses.

From these tentative assignments of dsRNA segments to specific viral proteins, the segments of particular interest in terms of strain differences appear to be 4, 5, 9 and 11. Segments 4, 5 and 9 being involved in host range determination and stimulation of neutralizing antibodies and segment 11 being involved in the serotyping by ELISA. In this context it is interesting to note that the segments that showed the greatest variability in the hybridization experiments were 4, 5 and 6. Segments 9 and 11 were often hard to evaluate due to a lack of separation from other segments for 9 or lack of probe copying for segment 11. The segments that seemed to show the greatest degree of homology between isolates were 1-3, 7, 8 and 10 which all code for either inner shell proteins or nonstructural proteins and hence are less important in terms of infectivity and host tropism and so would be expected to be conserved throughout all the isolates. Segments 4 and 5 were commonly missing from heteroduplex formation at even the lowest stringencies indicating that these segments are quite different (greater than 35%) in their genetic sequences between the various isolates. The hybridization of NCDV cDNA probe with local calf rotavirus RNA showed close similarity in seven of the eleven segments but was still quite different in segments 4, 5, 10 and 11. This indicates that the important segments, in terms of host range selectivity and infectivity are a lot more variable than the nonstructural and inner shell segments.

In reovirus it has been determined that the S_1 gene which codes for the O_1 outer capsid polypeptide, plays the key role in determining specific

virus-host cell interactions and is a major determinant of neurovirulence. Other genes also play a role in virulence possibly through control of viral replication rather than by determining virus-host interactions. The S_1 product is known to contain the neutralization and haemagglutination antigens. Similar mechanisms may operate in rotaviruses (this is developed further in the General Discussion).

It appears from the results of hybridization that segments 4 and/or 5 and possibly 9 are the key segments in determining host range. If this is in fact the case then it may be of value to screen rotavirus isolates with single gene probes (as described in 2.5 and 3.3) to segments 4, 5 and 9. This would allow a comparison of isolates at the level of the significant segments that effect the spread of rotavirus through populations and possibly across species barriers without the confusing data of the other segments. It would also allow the detection of major changes in these genes in prevalent types in the host populations and their significance at the clinical level.

Reassortment of rotaviral genes between strains has been shown to occur in vitro (Matsuno, et al., 1980) and is a likely mechanism to occur in vivo resulting in new strains of rotavirus. If the important genes in determining host range and virulence are 4, 5 and 9 then a reassortant with changes in one or more of these positions is likely to have a major effect on the epidemiology of the virus.

The relationship of the local rotaviral isolates at the genetic level is diverse. The main objective of these experiments was to determine how closely related the isolates obtained from different host species within the one geographical area and at the same time period were. If there is a high rate of cross transmission between species or a high rate of mixing of dsRNA segments, then upon hybridization of these different isolates there would be a high degree of relatedness. This in fact was not

observed. Although dog rotaviral cDNA was the only local isolate to be tested against the spectrum of isolates it was obvious that the different isolates form a heterogeneous group at the genetic level. Hybridization with the two cultivable rotaviruses, NCDV and SA_{11}, also showed that local isolates were quite different. There was no evidence obtained from these experiments that rotavirus isolated from different host species in the Dunedin urban and rural areas between 1978 and 1980 are any more than partially related. This relationship appears to correlate with genes that are likely to have no influence on the host range or virulence of the virus isolates but are probably coding for internal structural or nonstructural proteins. Therefore, between 1978 and 1980 there was a pool of relatively unrelated rotaviruses circulating in human and animal populations. This does not mean that these unrelated rotaviruses are restricted to certain hosts but no evidence was found of cross transmission. Sequence diversity of rotavirus isolates is not limited to rotaviruses from different host species. Street, et al., (1982), using the same hybridization procedure to compare human rotavirus isolates, also found a remarkable degree of sequence diversity. These two studies on hybridization of rotaviral genomes support the findings of RNA PAGE that show a high degree of genome diversity.

The hybridization technique used, known as the "Northern Blot" proved to be a very simple and efficient method of comparing the genetic relationship of rotaviral isolates. By using probes for single genome segments that are of particular significance in infectivity and virulence a clearer understanding of the epidemiology of this virus in human and animal populations could be made.

GENERAL DISCUSSION

It was known before this study was initiated that rotavirus was commonly found in young animals and it was of interest therefore to establish the degree of relatedness of human and animal rotaviral isolates and whether there was any possibility of natural transmission of rotavirus between different host species, especially whether humans could be infected with rotavirus from an animal source.

Althouth no direct evidence was found for transmission of animal rotavirus from animal to human hosts, a number of significant points were found in this study giving a greater understanding of the incidence of rotavirus in the local community. The genetic studies have yielded a greater understanding of the heterogeneous character of this group of viruses. Very briefly the significant conclusions determined from this study are:

1) That rotavirus is a very common, pathogenic agent in both man and a number of animal species, namely calves, foals, dogs, cats and pigs.

2) That rotavirus does infect both dogs and cats, previously unknown in New Zealand and not commonly reported overseas.

3) That animals may have a rotaviral infection without any clinical evidence of the disease.

4) That the ELISA system is very sensitive, reliable and rapid for screening large numbers of specimens of faecal material for rotaviral antigen and of sera for rotaviral antibodies.

5) That symptoms in animals can be as severe and debilitating as in humans, as was seen with foals and pups.

6) That antibody levels in young adults may indicate recurrent exposure to rotaviral antigen.

7) That recurrent exposure to rotavirus in young adults may not always be associated with contact with infants.

8) That PAGE showed that what was being identified as rotavirus by ELISA was in fact rotavirus as evidenced by the typical rotaviral genome migration pattern.

9) By PAGE, 15 human, 7 bovine, 1 equine, 1 canine and 1 feline electropherotypes were differentiated in local specimens.

10) There is a large degree of heterogeneity of electropherotypes within a host species as seen in both human and bovine isolates.

11) There is also a large degree of heterogeneity between isolates from different host species.

12) The difference in electropherotypes at interspecies level was no greater than at the intraspecies level.

13) There is no evidence of a consistent species or geographical electropherotype.

14) Cat and dog rotavirus isolates were electropherotyped for the first time in the world.

15) The New Zealand occurrence of rotavirus and the range of electropherotypes resolved is consistent with similar studies done overseas. One difference from a number of other studies is the antibody levels detected in young adults.

16) Hybridization by the "Northern Blot" technique proved to be useful in determining the degree of genetic similarity between different rotaviral isolates.

17) Of the cultivable rotaviruses, SA_{11}, NCDV and NI were more closely related to each other than to the human derived WA virus.

18) The SA_{11} rotavirus is not closely related to local human and animal isolates showing no greater than 53% similarity with most segments.

19) The two cultivable bovine rotaviruses NCDV and NI are identical by hybridization comparisons.

20) NCDV shows a high degree of relatedness with local calf isolates except in band 4.

21) The local dog rotavirus isolate is not closely related to any other animal or human isolates by hybridization studies.

22) The single band probe showed that hybridization occurs specifically between equivalent bands and is not a random process.

23) Segments four and five were the most variable by hybridization with segment five sometimes showing less than 50% homology between isolates.

24) As a whole the rotavirus isolates were very heterogeneous in their genomes when compared by hybridization studies.

From these conclusions it can be seen that rotavirus is a common infectious agent of both man and animals but its epidemiological pattern is likely to be very complex because of the heterogeneous pool of virus types that make up these infections. A better understanding of the epidemiology of rotaviral infection is dependent on the answering of a number of questions that are still unresolved; these include:

1) How stable genetically and antigenically is any particular rotavirus type?

2) Do rotaviruses undergo "drift" and "shift" changes leading to minor and major changes in serotypes and virulence, as with influenza viruses?

3) Which genetic segments are important in determining virulence and host tropism?

4) How much change can there be in these particular segments before virulence and host tropism is effected?

5) Although reassortment of gene segments has been demonstrated <u>in vitro</u> does this occur naturally <u>in vivo</u>?

6) Do rotaviruses infect the respiratory route as has been suggested by Holdaway, et al., (1982)?

It is in these areas that future rotaviral research needs to be directed.

Presently there is a lot known about the different characteristics of rotavirus and rotaviral infection but the various pieces have not been drawn together to show how rotaviruses spread, change or cross species transmit. It is known there are at least three human serotypes, possibly five, and yet there are probably dozens of different electropherotypes. The relationship of a change in electropherotype to the stability of serotypes is not presently known or how great a change there has to be to effect a change in serotype. Also it is not known whether the antigens that determine serotyping, as determined at present, are in fact important in determining host tropism or virulence.

One of the factors that has hindered the comparison of types and a full understanding of the relationship of gene expression to host tropism and virulence is the inability to culture rotavirus isolates in vitro. The isolates that have been adapted to grow in tissue culture do not yield large amounts of virus and consequently a lot of culturing is needed to obtain quantities of virus in sufficient amounts to conduct required experiments. The finding that addition of trypsin to the culture media initiating greater yields of virus (Babiuk, et al., 1977) was a major step forward but present production of virus still limits the work that can be done at the genetic level and particularly the understanding of genetic expression in whole virus. The development of restriction enzyme technology and of receptive bacterial hosts for viral genes has meant that viruses, previously unable to be studied at the translation and transcription levels can now be studied within the bacterial system. Viral genes and other expressed products can be produced in large amounts by cloning genes within a bacterial

host. If such technology is applied in rotaviral research a greater understanding of the biological relationship of different genetic types may be resolved.

One of the limiting factors in the understanding of the relationship of genetic material, the expressed gene product and the effect of genetic variation, is the lack of agreement of how many rotaviral gene products there are, their coding and the function of these products. Presently there are just about as many coding systems for rotaviral polypeptides as there are reports on their characterization. There is also a lack of agreement on the function of these polypeptides. Various methods have been used to characterize rotaviral gene products -- from studying purified virus, or cell culture products, to serological studies of individual protein components (Espejo, et al., 1981; Thouless, 1979; and Bastardo, et al., 1981). The coding system and number of protein products most commonly agreed upon is as used by Thouless (1977). However, Espejo, et al., (1981) in studies on the effect of trypsin on structural polypeptides of SA_{11} virus concluded that only 5 of the structural polypeptides were primary gene products as opposed to 8 or 9 as found by most other groups. They concluded that polypeptides O_1 and O_3 (equivalent to VP_5 and VP_8 by their coding system) were in fact trypsin cleavage products from polypeptide I_3 (VP_3). They also had doubts as to the existence of a reported structural polypeptide VP_4, this they thought was a cleavage product produced by chymotrypsin. Trypsin is routinely used in tissue culture of rotaviruses to increase yields and chymotrypsin is a common contaminant of trypsin preparations. These results are in disagreement with the majority of reports to date but, however, cannot be ignored. If such a cleavage did occur then it may be analagous to that observed for HA proteins of myxoviruses where cleavage activates the infectivity of these viruses probably at the level of penetration (Chappin and Scheid, 1980). Mason, et al., (1980) found that viral structural

polypeptides VP_4 and VP_5 were not synthesized *in vitro* as primary gene products. They, too, believed that further processing must occur for the production of these structural polypeptides. In a study by Smith, *et al.*, (1980) where separated dsRNA segments were translated it was reported that the first four segments produced polypeptides corresponding to I_1 through to I_4, segment five coded for polypeptide O_1 and segment six for polypeptide I_5. In later reports from this same group (Bastardo, *et al.*, 1981) I_5 has been changed to I_4 and what was I_4 in earlier reports no longer appears to exist as a primary gene product. Probably two key reasons exist why reports of gene products so commonly disagree between *in vitro* production and characterization of viral polypeptides as compared with characterization of polypeptides from purified virions. Firstly, in the *in vitro* systems there is no further processing of viral polypeptides occurring such as glycosylation and it is known that at least 2 are glycosylated (O_2 and O_4); also nonstructural polypeptides will be produced that will not correlate with characterization of purified virus particles. Secondly, with purified virus the actual methods used for growth and purification may effect the viral polypeptide, as has been suggested with trypsin, this too would bring disagreement with *in vitro* systems. Comparisons of single and double shelled particles can never fully guarantee purification of the two types and again methods to purify or produce single shelled particles may produce an uncharacteristic polypeptide characterization. An agreement on the coding of rotaviral polypeptides and a clarification of which are in fact primary gene products would indeed be welcomed.

A recent publication by Bastardo, *et al.*, (1981) has thrown some light on the role various substructures of rotavirus particles may play in infection and protection. By separating out viral polypeptides by electrophoresis and then producing specific rabbit sera against individual polypeptides they were able to investigate which substructures were important in

inducing neutralizing antibodies, haemagglutination inhibition antibodies, group and type specific antibodies. Antisera produced in this manner provided the first evidence that two substructures O_2 (equivalent to their gp34) and O_3 (p26) of SA_{11} rotavirus can induce production of type-specific neutralizing antibodies in rabbits. Polypeptide O_2 (gp34) was also capable of inducing the production of haemagglutination-inhibiting antibody, this polypeptide represents the major outer shell component of the virus. Antisera raised against O_2 (gp34) and O_4 (gp25) of SA_{11} virus reacted with double-shelled particles of both SA_{11} and NI calf rotaviruses indicating that there are cross reactive antigenic determinants present in the outer capsid of the virus. Antisera to polypeptide O_1 (p62) however reacted only with the homologous double-shelled particle. Results also indicated a group-specific antigenic determinant on the inner capsid which was best detected with antisera to I_4 (p42), this polypeptide surprisingly, also showed a slight neutralizing activity for SA_{11} that was type specific. Polypeptide I_4 is the major inner shell protein of rotavirus particles. Two polypeptides of rotavirus, O_2 and I_4, show evidence of both common and type-specific antigens being present on a single protein. This is not unique as it has been shown previously for the influenza virus haemagglutinin and for herpes simplex virus types 1 and 2 (Bastardo, 1981). From these results 5 polypeptides of rotavirus particles would appear to be of particular importance, O_2 and O_3, in producing type specific neutralizing antibodies, O_1 in producing a type specific antibody, O_2, O_4 and I_4 in producing cross reactive antibodies.

The dsRNA segments that code for these polypeptides are thought to be segment 5 (O_1), segment 6 (I_4), segment 9 (O_2), segment 10 (O_3) and segment 11 (O_4). These results are in agreement with Kalica, et al., (1981) who considered that the product of segment 5 was important in determining

cell tropism and hence possibly host range. Greenberg, et al., (1981) considered that segment 9 coding for polypeptide O_2 was likely to be important in producing neutralizing antibodies. From our results of hybridization experiment at segments 4 and 5 were particulary variable showing little homology between isolates. Segment 9 also appeared to be fairly variable although this was often difficult to detect as segments 7, 8 and 9 usually travelled closely together upon electrophoresis. This variability was shown between different host species in our study but such variability also occurs between different rotavirus isolates from human hosts as has been shown in similar hybridization studies by Street, et al., (1982). If sequence variability in these important segments had been restricted to interspecies comparisons it may have indicated that there was a true host range restriction controlled by certain antigenic determinants, but because such variability also occurs in isolates from one host species it suggests that rotaviruses as a whole are a very heterogeneous group. Indications from the work of Street, et al., (1982) and Rodger, et al., (1981) and Espejo, et al., (1980) are that this heterogeneity is not a static thing but that rotaviruses may be continually changing their genetic makeup either through minor changes by point mutations or by major changes through reassortment of genetic segments, similar to "shift" mechanisms in influenza viruses.

It has been observed in a number of crosstransmission studies that immunization of an animal with one type of rotavirus does not give that animal full protection when challenged with rotavirus from a different host source. It has been noted that the infection with the second virus is reduced in the severity of the symptoms produced but that virus is still excreted in the faeces indicating that virus infection and multiplication is occurring. When this second virus is given to a control animal that has not been previously immunized the infection normally causes typical symptoms of a rotaviral infection. This means that it is not just a dosage

effect in the immunized animal but that for some reason there is a partial protection produced to reinfection with another rotavirus type (Tzipori, et al., 1980; Woode, et al., 1978). The problem is to explain why there is only a partial protection occurring. The theory of neutralizing antibody action is that it binds to antigenic determinants of the virus, inhibiting absorption of the virus particle to receptors on the surface of susceptible cells and consequently inhibiting any infection. Such a mechanism has to be an "all or none" effect, either the antibody does inhibit adsorption and penetration of the virus or it doesn't. Neutralizing antibody can have no effect on the virus infection once it has gone beyond the point of penetration. Therefore, the activity of neutralizing antibody alone does not explain the situation where infection is occurring but the severity of the disease is markedly reduced. One possible explanation is that upon initial infection with rotavirus, antibody is produced that neutralizes that particular type of virus. Upon challenge with a second rotavirus type this antibody only neutralizes one antigenic determinant, of say the O_2 polypeptide but has no effect on the antigenic determinants of the O_3 polypeptide. This may mean that the virus is still able to absorb to a cell receptor but the receptor may be on a different type of cell which the virus then penetrates and replicates in but destruction of this cell type does not produce any severe symptoms of diarrhoea. There are some reports of complete cross protection between rotaviral types (Tzipori, et al., 1980) and presumably this is when neutralizing antibody to one type completely blocks the important antigenic determinants of the challenge rotavirus. There is a certain amount of histological evidence for such a theory. Middleton, et al., (1975) reported that when human rotavirus was inoculated into gnotobiotic piglets that virus only infected the duodenal mucosa of the proximal portion of the small intestine as evidence by areas of villous atrophy and by indirect immunofluorescence of rotaviral antigen in mucosal

columnar epithelial cells. However, in a number of fatal human cases immunofluorescence extended throughout the small intestine including the distal ileum. In a study by Thiel, et al., (1978) pig rotavirus was inoculated into gnotobiotic piglets producing clinical symptoms of diarrhoea, anorexia and depression. Upon histological examination it was found that damage extended throughout the small intestine and in scattered foci in the colon but was mainly found in the jejunum and ileum. Villous atrophy and elongation of the crypts of lieberkühn were very evident. It was suggested by this group, as had been previously by Snodgrass, et al., (1977) that villous atrophy can result from the direct destruction of the villous columnar epithelial cells resulting in a rapid replacement of damaged epithelial cells and the presence of immature cells on the villi tips. This would lead to reduced absorption and decreased digestive enzyme activity resulting in a nett fluid loss. Immunofluorescence examination of infected villi by both Snodgrass, et al., (1977) and Thiel, et al., (1978) suggest that only the apical half of the villi are infected rather than cells in the crypts and that as the infection progresses only the very tips of villi are infected. It appears that upon infection with rotavirus that produces severe symptoms of diarrhoea that damage to villi extends throughout the small intestine and possibly into the colon. However, with a subclinical infection damage appears to be much more restricted and only in certain areas of the small intestine. It may be that in subclinical infections virus is infecting a type of cell of much smaller population in the small intestine represented by different receptors on the cell surface.

Such a mechanism of infection would be somewhat analagous to that of reovirus where types 1 and 3 can be differentiated on the existence of a different σ_1 protein. This protein is the major determinant of neurotropism. Reoviruses with a type 1 σ_1 will adsorb to receptors on ependymal cells when inoculated intracerebrally into newborn mice whereas virus with type 3 σ_1 will adsorb to receptors on neuronal cells causing a much more

severe infection. The σ_1 polypeptide produces specific neutralizing antibody and is also the haemagglutinin in reoviruses (Weiner, et al., 1977). This is very similar to the O_2 polypeptide of rotaviruses which produces neutralizing antibody and is also the haemagglutinin. It may be that this structural protein of the outer capsid is key in determining what type of intestinal cell is infected and consequently the severity of the disease produced.

Further research on rotavirus needs to be directed towards the development of techniques for cloning rotaviral genes in bacterial systems. This would allow a greater flexibility in handling the individual genetic segments and the translated gene products. In being able to handle these genes and the protein products individually it will allow for a better understanding of the direct relationship of genetic sequence to protein product and subsequent antigenic determinants. Particular study on gene segments five and nine would most likely lead to an increased understanding of the effects of variable genetic make up to the subsequent effect on cell tropism and virulence of different rotaviral isolate types. It will not be enough to study the genetic relationship of different isolates, whether by PAGE or hybridization, until it is understood which differences at the genetic level are in fact significant and how such changes effect the infectivity and virulence of the virus. Once this relationship is understood then epidemiological studies based on the genetic character of virus isolates will be a lot more meaningful in terms of deducing which are the virulent types to be watched for. Also, once the important genes have been determined for infectivity and virulence, these particular genes can be monitored by hybridization techniques as have been outlined in this thesis to see if they are being reassorted into other rotaviral types in the natural environment.

At present the large number of reports on different genetic types being isolated is more confusing than revealing. It is likely that only

1 or 2 of the genes are important in infectivity and virulence and studies on the incidence of these particular genes in isolates may yield much more information than data on all the gene segments which may only cloud the epidemiological patterns of rotavirus in the community. Tracing of individual gene segments by PAGE is not adequate to show true degrees of relationship of independent genetic material, only hybridization comparisons or sequencing will give the required information. The technique used for hybridization studies in this thesis is useful for such studies. The fact that genetic material can be transferred to DBM paper and stored at -70°C means that a library of rotaviral isolates can be built up that can be compared a number of times.

Until the epidemiology of rotavirus is understood and the important gene products resolved, attempts at vaccine production are pointless. However, if there is a restricted number of virulent genes then these genes may be cloned and translated in bacterial systems for specific vaccine production. This has to be the overall aim of rotaviral research as long as millions of this world's children are dying from rotaviral infection.

Future research on rotaviruses that only adds to the data on the incidence of infection in either human or animal populations or reveals the number of electropherotypes present in a local community over a period of time is of very little value in the light of present knowledge and the yet unanswered questions. Such studies are admittedly of interest to the particular investigators, revealing their own local problem, but rarely does this differ from the large number of reports from throughout the world. It is very clear that we are dealing with a heterogeneous population of viruses that are highly infectious and are a severe problem in the young. Repetition of such data is largely meaningless. Over the past four or five years the bulk of rotaviral research, this study included, has been aimed at the characterization of the virus itself. Such studies have been

of value in determining the heterogeneity of the rotavirus group but on their own contribute little and may even confuse the issue of the spread and control of spread of the virus through a population. Already there are positive indications that rotaviral research is heading more in the direction of correlating rotaviral variability with the immune status of the host. The question seems to be no longer how variable are our isolates but what does this variability mean in terms of function, infectivity and virulence? Ideally there needs to be definite objectives made on what direction rotaviral research should take in the light of the problems it causes and what needs to be known to resolve those problems. Funding and research planning could then be made in the light of such objectives and rotaviral research would be heading in a definite direction. In the next few years there needs to be research into: the technology for cloning and sequencing particular rotaviral genes; a greater correlation of genetic, polypeptide diversity with their corresponding biological functions; and the stability of rotaviral types and their introduction and replacement in host populations. Most of all there needs to be correlation of the data that is being produced worldwide.

Over the past decade there has been a lot of progress made on the understanding of the causative agents of infantile gastroenteritis, there is still much to be learned and applied. Meanwhile thousands of children will die from rotaviral infections so there is no room for complacency in the search for answers.

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ABSTRACT

Techniques for detecting rotaviral antigen in faeces and human rotaviral antibodies were developed so as to ascertain the frequency of rotaviral infections in humans and animals. Of the four techniques used: electron microscopy (EM), radioimmunoassay (RIA), staphylococcal protein A agglutination, and enzyme linked immunosorbent assay (ELISA), ELISA proved to be the more suitable. The ELISA system was a rapid method of screening a large number of specimens while retaining sensitivity and accuracy. It was able to detect purified SA_{11} rotavirus to a level of 15ng in 25µl volume. Overall, 425 specimens were assayed of which 148 (34.7%) were rotavirus positive. These came from human, feline, canine, bovine, equine, and porcine individuals. Feline and canine species were of particular interest because of their frequent contact with humans, both showed relatively high infection rates, 27.8 and 18.6 percent respectively. Of the total number of specimens that were rotavirus positive only 52 percent came from individuals that had symptoms of a rotaviral infection. Antibody studies in humans (20-23 years) indicated that rotavirus is a common infectious agent of young adults. These results indicated that domesticated animals and young adults may be major sources of infectious rotavirus for children in the 6 month to 2 year age group for whom rotavirus infection is so dangerous.

From these positive specimens, 48 were electropherotyped by polyacrylamide gel electrophoresis. This revealed 15 different electropherotypes of which 7 were human; 5, bovine; 1, equine; 1, canine; and 1, feline. There were no electropherotypes that were common to more than one species of origin. The variability of electropherotypes from a single species was high and as great as variability observed for electropherotypes compared between different species of origin. Some electropherotypes from different animal species were very similar. The different electropherotypes resolved from human and bovine hosts showed no signs of a sequential pattern of appearance or of genetic reassortment.

Molecular hybridization techniques using complementary DNA labelled with ^{32}P and rotaviral RNA bound to diazobenzylaxymethyl paper further investigated the degree of relationship of rotaviral isolates. Two of the cultivable rotaviruses, NCDV and NI proved to be very similar if not identical at the genetic level with no greater than a 10% difference in base matching. The SA_{11} cultivable isolate also showed a high degree of relationship to NCDV in 4 of its segments. However the cultivable human isolate WA, was not closely related to any of the other cultivable rotaviruses. NCDV also showed a high degree of relatedness to local bovine rotavirus isolates (90% homology) in 5 of the dsRNA bands. Segments 4, 5, 6 and 9 showed the greatest variability between isolates. These segments may be significant in determining serotypes, host range and virulence. The segments that showed the greatest degree of homology between isolates 1-3, 7, 8 and 10 all code for either inner shell proteins or nonstructural proteins. Hybridization using cDNA made to segment 2 only, showed that hybridization was a specific and not a random process.

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APPENDIX I

REAGENTS AND MEDIA

1. PBS, pH 7.2

NaCl	8gm/l
K Cl	0.205gm/l
Na_2HPO_4	1.135gm/l
KH_2PO_4	0.2gm/l

2. Tissue Culture Medium for Primary Monkey Kidney and BSC Cells

2.1 Growth Medium

M199 (GIBCO)	0.99gm
Heat inactivated FCS	10ml
4.4% $NaHCO_3$ solution	5ml
Gentamycin (80mg/2ml)	0.1ml
150mM Hepes buffer	1ml
Double distilled water	80ml

2.2 Maintenance Media. Same as growth medium except only 5% of FCS (heat inactivated) and 5µg/ml trypsin were added for rotavirus growth. The medium was sterilized by filtration.

3. Medium for Madin Darby bovine Kidney Cells (MDBK)

3.1 Growth Medium

MEM (GIBCO) with non-essential amino acids	0.96gm
Heat inactivated FCS	10ml
4.4% $NaHCO_3$ solution	5ml
Gentamycin (80mg/2ml)	0.1ml
1% non-essential amino acids (100 x conc.) (GIBCO)	0.1ml
Double distilled water	80ml

3.2 Maintenance Medium. Same as growth medium except only 5% of FCS (heat inactivated) and 5µg/ml trypsin were added. The medium was sterilized by filtration.

4. Iodination Reagents

4.1 Phosphate buffer, pH 7.5

A. 0.25M Na_2HPO_4 (17.75gm/500ml)

B. 0.25M NaH_2PO_4 (7.8gm/200ml)

Add B to A until pH 7.5 is obtained.

4.2 Chloramine T (prepare freshly before use)

Chloramine T	0.035gm
0.25M phosphate buffer, pH 7.5	10ml

4.3 Sodium Metabisulphite solution

Sodium metabisulphite	0.024gm
0.25M phosphate buffer, pH 7.5	10ml

5. ELISA Reagents

5.1 PBS-Tween 20

Tween 20	0.5ml
PBS, pH 7.2	1 l

5.2 Citrate-Phosphate buffer, pH 5.0

A. 0.1M citric acid (2.1gm/100ml)

B. 0.2M Na_2HPO_4 (14.33gm/200ml)

Add A to B until pH 5.0 is obtained.

5.3 H_2O_2 substrate (prepare freshly before use)

Citrate-phosphate buffer, pH 5.0 (fresh)	10ml
1/100 dilution of H_2O_2 (30%)	0.2ml
orthophenylene diamine	8mg

Keep out of light. This reagent is also potentially carcinogenic.

6. Antibody-HRP Conjugation Reagents

6.1 0.3M $NaHCO_3$, pH 8.1

$NaHCO_3$ 2.52gm/100ml

6.2 1-fluoro,-2-4, dinitrobenzene

0.1gm in 10ml absolute ethanol

6.3 0.08M $NaIO_4$

1.71gm in 100ml distilled H_2O

6.4 0.01M Na_2CO_3

Na_2CO_3 1.06gm/1 l

7. Slab PAGE Reagents

A. Stock Solutions

7.1 Acrylamide 30%

Acrylamide 30gm

N_1N^1-methylene-bis-acrylamide 0.8gm

Add distilled H_2O to 100mls. Filter sterilized and stored in dark.

7.2 Lower Gel Buffer

Tris (hydroxymethyl) Methylamine 18.2gm

Sodium lauryl sulphate 0.4gm

Add distilled H_2O to 100ml and adjust pH to 8.8 with HCl.

7.3 Upper Gel Buffer (stacking gel)

Tris (hydroxymethyl) Methylamine 61.0gm

Sodium lauryl sulphate 0.4gm

Add distilled H_2O to 100ml and adjust pH to 6.8 with HCl.

7.4 Ammonium Per Sulphate (prepare freshly before use)

Ammonium per sulphate 1gm/10ml

7.5 Sample Buffer

Upper Gel buffer	30ml
Bromophenol blue (0.1% in ethanol)	1.5ml
Glycerol	15ml
Sodium lauryl sulphate	3.0gm
2-mercaptoethanol	1ml

Add distilled H_2O to 100ml and store in airtight container.

B. Formation of Gels

7.6 Lower Gel (10% acrylamide)

lower gel buffer	10ml
acrylamide stock	13.4ml
distilled H_2O	16.6ml
10% ammonium per sulphate	0.3ml
$N_1N_1N^1{}_1N^{11}$-Tetra Methyl-ethylene-diamine (TEMED)	0.03ml

Pour between plates to within 2.5cm from top. Overlay with H_2O til gel has set.

7.7 Upper Gel (5% acrylamide)-stacking gel

Upper gel buffer	2.5ml
acrylamide stock	1.67ml
distilled H_2O	5.87 ml
ammonium per sulphate	0.09ml
TEMED	0.03ml

Pour on top of set lower gel and insert comb for forming wells.

7.8 Electrophoresis Buffer, pH 8.3

Tris (hydroxymethyl) methylamine	15.5gm
Glycine	72gm
Sodium lauryl sulphate	5mg
distilled H_2O	5l

Adjust pH to 8.3 with HCl.

8. Phenol Extraction of Double-Stranded RNA from Virus Particles

8.1 0.01M Sodium Acetate Buffer, pH 5.0

Sodium acetate	0.34gm
6% (v/v) acetic acid	0.75ml
NaCl	0.73gm
Ethylenediamine tetra-acetic acid	0.11gm
distilled H_2O	250ml

8.2 90% (v/v) Phenol

liquified phenol (60°C)	90ml
0.01M acetate buffer, pH 5.0	10ml

8.3 Extraction of dsRNA

1. Suspend virus particles in 2ml 0.01M sodium acetate buffer and add 0.02gm SDS.

2. Add 2.5ml 90% (v/v) phenol at 60°C. Incubate at 60°C for three minutes, shaking every 30 seconds.

3. Cool the extract on ice. Separate the aqueous and phenol phases by centrifugation at 4,000 g for 5 minutes. Remove the aqueous (upper) phase.

4. Re-extract the aqueous phase twice more with 1.5ml and 1ml of 90% phenol. Incubate at 60°C for 3 minutes on each occasion, and cool on ice.

5. Remove the dissolved phenol from the final aqueous phase by extracting three times with ether. Discard the ether (upper) layer each time.

6. Precipitate the RNA by adding two volumes of sodium chloride-saturated ethanol. Store at -20°C for 1-2 hours or overnight.

7. Pellet the RNA by centrifugation at 4-5,000 g for 10 minutes. Wash once with ethanol and once with ether. Dissolve RNA in PAGE sample buffer.

APPENDIX II

SEQUENCE RELATIONSHIPS BETWEEN THE GENOME SEGMENTS OF HUMAN AND ANIMAL ROTAVIRUS STRAINS

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Running Title:

Human and animal rotavirus strains

ABSTRACT

The sequence relationships of a range of cultivable and noncultivable human and animal rotaviruses have been investigated by hybridisation of rotavirus cDNA probes to genomic RNA's immobilised on diazobenzyloxymethyl (DBM)-paper. Under conditions of low stringency (34 percent base-mismatch tolerated) most genome segments exhibit partial homology except for genes 4 and 5. In contrast, under more stringent conditions of hybridisation where no more than 8% base-mismatch is tolerated, few segments exhibit homology. Generally the human and animal rotaviruses have been found to possess distinct nucleic acid sequences that exhibit only a low order of sequence relatedness. These results are consistent with the notion that both cumulative changes in nucleic acid sequences and the interchange of segments may be involved in the evolution of distinct rotavirus strains.

INTRODUCTION

The rotaviruses are not only important aetiologic agents of diarrhoeal disease in man but also are of widespread occurrence amongst animal populations. Rotaviruses have been recorded in bovine (18), equine (10), porcine (22) and canine populations (7) and virus has been isolated from a number of other animal species (12).

In the accompanying paper (24) a technique has been developed which enables the sequence relationships between different rotavirus RNA's to be investigated. The procedure is based on the hybridisation of cDNA probes specific for the RNA of a particular rotavirus to the viral genomic RNA which has first been resolved by polyacrylamide gel electrophoresis and then immobilised by blotting onto diazobenzyloxymethyl (DBM) paper. Varying degrees of sequence relationship were detected amongst the human strains which were studied by this method. In some instances evidence was obtained for the absence of sequence relationship between a single human rotavirus gene and cDNA probes copied from genes of similar viruses isolated at a comparable time. Other human isolates showed evidence of major sequence differences in most of the RNA segments, implying that the nucleic acids of these strains have a distinct origin.

The reoviridae in general (4) and the rotaviruses in particular (15) are known to interchange segments at a high frequency under suitable conditions in culture. An attractive explanation for the evolution of distinct strains might therefore be based on the interchange of segments between the various animal species which act as reservoirs of infection. Interchange of genes in this fashion has been suggested as a possible mechanism for the evolution of new strains of orbivirus (11, 27). Furthermore, reassortment of genes is a likely mechanism for the development of new influenza A virus strains (21): the H3 subtypes, when compared to pre-existing subtypes, exhibit entirely different nucleic acid sequences

for their haemagglutinin genes (3). These genes are thought to have originated from the avian reservoir (17, 21).

This communication reports the results of a series of cross hybridisation analyses in which the extent of sequence relationships between a series of cultivable and wild-type rotavirus strains have been investigated. The aim of the work has been to search for evidence of sequence identity or relatedness between rotavirus genes of different origin. The results obtained imply that interchange of genomic segments may have occurred during the evolution of these viruses and that the genomes examined have been subjected to major sequence divergence during the evolution of this group of viruses.

MATERIALS AND METHODS

Virus and RNA - Cultivable rotaviruses were propagated as described elsewhere (24) and the genomic RNA extracted. Wild strains of rotavirus were purified from faecal material by the method of Croxson and Bellamy (6) and the RNA extracted by detergent lysis. The method of Rodger and Holmes (23) has been adopted to described the source and year of isolation of these strains.

Synthesis of cDNA probes and transfer of RNA to diazobenzyloxymethyl (DBM) paper - The method of Street, et al., (24) was used. For the synthesis of the canine rotavirus probe, the double-stranded RNA was first purified by isopycnic sedimentation on C_SSO_4/Eth Br gradients (24).

Hybridisation of cDNA probes to immobilised RNA - Essentially the method of Street, et al., (24) was followed. A mixed cDNA probe specific for the RNA of a particular rotavirus strain was hybridised to other rotavirus RNA's which had been immobilised on DBM paper. This method enables the fraction

of a genome segment exhibiting homology to be estimated by comparison with the radioautograph of the corresponding homologous hybridisation. The sequence relatedness of that portion of the genome which exhibits homology can then be determined by carrying out hybridisation at different stringencies following adjustment of the formamide concentration (1). Hybridisation at 52^{0} in the absence of formamide detects sequences which possess up to 34 per cent base-mismatch whereas hybridisation in the presence of 50 per cent formamide detects sequences having only 8 per cent base-mismatch or less (24).

RESULTS

Diversity of gel electrophoretic profiles of rotavirus RNAs - Figure 1 presents a typical result of an electrophoretic analysis in which a series of animal and human rotavirus RNAs were resolved on the same polyacrylamide gel. It is clear from Figure 1 that a number of differences can be detected when the migration rates of the individual segments are compared. This

- Figure 1 near here) -

result confirms the observations of previous investigators (9, 13, 14, 23, 26) that the 'electropherotype' of the viral RNA is a distinctive feature of rotavirus isolates. However certain isolates (NCDV, lane 1; NI, lane 2) are not distinguished from each other by this procedure whereas some of the other bovine isolates (for example BO/DUN/25/79 and BO/DUN/70/79) are able to be distinguished on the basis of the mobility of segments 7, 8 and 9. Other genes (for example gene 5) show considerable variation in electrophoretic mobility.

Sequence relationships of selected rotaviruses with Simian-11 virus cDNA probes - The series of rotavirus RNA's shown in Figure 1 and other comparable series from separate acrylamide gels were transferred to DBM paper by

transverse electrophoresis (24). The immobilised viral RNA's were then probed with cDNA sequences specific for all eleven SA-11 virus genes. Figure 2a presents the results obtained when the hybridisation was carried out under low stringency in the absence of formamide. Hybridisation occurred between the SA-11 probe and many of the rotavirus genes of the

- (Figure 2 near here) -

strains examined. Two exceptions are obvious: genes four and five show little evidence for homology with the SA-11 genome under these conditions of hybridisation which should detect sequences which on average have base mismatches as great as 34 per cent (examples of missing bands are arrowed in Figure 2a). It may be concluded that cDNA probes specific for genes four and five of SA-11 show little or no relationship with genes four and five of the other strains examined. This was confirmed by hybridisation at a lower temperature (37^{o}) which also failed to detect homology (data not shown). Overall the homology detected was generally low when compared to the homologous SA-11/SA-11 hybridisation (channel 3 of Fig. 2).

Sequence relationships with bovine and canine isolates - Figures 3 and 4 present the results of a series of hybridisations similar to those presented in Figure 2. Probes specific for NCDV genes and those of the wild-type canine virus strain CN/DUN/59/80 were prepared and applied to the DBM-immobilised genome RNA's at both high and low stringency. For the NCDV

- Figures 3 and 4 near here) -

probes at low stringency (Fig. 3a) it can be seen that gene 5 again exhibits little evidence for homology with the equivalent gene of human, canine, equine or simian origin. Homology was evident however between the cDNA probe and gene 5 of other bovine isolates. It is also notable that the NCDV

probe showed some homology with SA-11 gene 4 (confirming the relationship already established in Fig. 2) but none with the human, nor with the wild-type bovine isolates examined (arrowed Fig. 3a).

When the stringency of hybridisation conditions was increased, few non-bovine genes showed homology with the NCDV probe. A notable exception for the series shown in Figure 3b are genes 6-11 of the Simian-11 virus which demonstrate strong homology. This implies that NCDV exhibits considerable sequence relatedness of these genes of this simian strain but not to genes 1-5. This relationship is less evident but detectable in the reciprocal high stringency hybridisation shown earlier (Fig. 2) in which a SA-11 probe was hybridised to the immobilised NCDV genome.

Figure 3 also demonstrates that the Northern Ireland bovine rotavirus (NI) and the Nebraska strain (NCDV), despite their diverse geographical origin, exhibit very close sequence relationships.

A similar cross-hybridisation analysis was carried out on the same series of isolates using a cDNA probe copied from the RNA of the wild-type canine strain (CN/DUN/59/80 (Figure 4a). Of the various gene 5 bands, only the equine gene showed homology with the corresponding canine probe. Gene 4 of NCDV, NI and EQ/DUN/1/79 exhibited homology with the probe but not gene 4 of the human cultivable strain (arrowed in Fig. 4a). At high stringency (Fig. 4b) few sequences of near-identity were revealed, confirming that the CN/DUN/59/80 isolate shares few regions of sequence identity with the other strains examined.

Sequence relationships between human and bovine rotaviruses - In Figure 3b there is some evidence that small regions of the NCDV genome share sequences of near identity with those of certain human isolates (see channels 14-17 of Fig. 3a and b). To determine whether the bovine rotavirus genome exhibits homology with a wider selection of human rotavirus genomes, an

an NCDV probe was hybridised to a series of human isolates (24).

- (Figure 5 near here) -

Figure 5 reveals that certain human isolates indeed contain minor sequences closely related to those of the bovine virus. This is particularly evident for segments 2 or 3 of the 'short' 1978 isolates. It is also evident from Figure 5b that segment eleven of the 'short' human genomes and segment 10 of the 'long' genomes also possess small regions of closely related sequence.

DISCUSSION

By analogy with influenza viruses (17) both antigenic 'shift' and 'drift' might be anticipated to occur amongst the rotaviruses in view of their known ability to interchange genome segments during mixed infections of cultured cells (15). However, in an investigation of sequence relationships between a relatively small number of field isolates, it is unlikely that definitive evidence for interchange of genome segments would be found. Segment interchange, nevertheless, is an attractive hypothesis to explain the results obtained here, since it would account for the lack of detectable homology observed for genes 4 and 5 in strains which at the same time demonstrated homology in most other segments. Antigenic 'drift' could account for the differences between those isolates which exhibit some base sequence homology but which are shown to be quite distinct when hybridisations are carried out under more stringent conditions.

The serological relationships existing between various rotavirus strains are yet to be well characterised because of difficulties encountered with the serological typing of this group of viruses (2, 32). Furthermore, nucleic acid homology need not necessarily lead to antigenic similarity and vice-versa. The rotaviruses possess a segmented genome and thus only a few

segments are likely to code for important antigenic proteins. Those segments which are antigenically relevant may nevertheless hold only minor sequences in common since the antigenic sites of proteins may involve only a portion of the gene (for example see ref. 28). Thus there may be little correlation between nucleic acid sequence homology and antigenic properties of rotaviruses.

Despite the absence of a direct relationship between serological type and nucleic acid sequence, the study of sequence homology between strains can be of value in two other ways. First, it may enable particular properties of the virus to be correlated with the presence or absence of particular genome segments. For example, most of the hybridisation evidence presented here is consistent with the notion that genes four and five may have some degree of species specificity: this does not appear to be absolute since genes 4 and 5 of the equine isolate showed sequence homology with the canine probe and equine gene 4 also showed homology with SA-11 and bovine probes. Kalica, et al., (15) investigated the properties of recombinants formed between bovine and human viruses and also concluded that genes 4 and 5 may be involved in the ability of the virus to grow in particular cell types.

Second, a knowledge of the sequence relationships between different rotaviruses might enable the relative importance of 'shift' and 'drift' in the evolution of these viruses to be assessed. Mechanisms that might be proposed to account for the evolution of the rotaviruses need to account for the complex and generally low order of sequence relationship demonstrated here. The common morphology (12), common antigens (31) and ability to cross species barriers (19, 20) all argue in favour of a common evolutionary origin for these viruses. A combination of segment interchange and progressive evolutionary divergence would thus account well for the unexpected low order of sequence relationship that we have found between human, simian, bovine, canine and equine rotaviruses.

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Council of New Zealand, the National Child Health Research Foundation and the World Health Organisation. We thank the Invermay and Wallaceville Research Centres of the Ministry of Agriculture and Fisheries for provision of faecal material containing rotaviruses of bovine and equine origin. Reverse transcriptase enzyme was provided by Dr. J. Beard of Life Sciences Inc., St. Petersberg, Florida in conjunction with the U.S. National Cancer Institute. We thank Professor R.E.F. Matthews for valuable criticism of the manuscript.

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LEGENDS TO FIGURES

Figure 1: Electrophoretic analysis of the genomes of different rotavirus strains. The RNA of cultivable and non-cultivable rotaviruses was applied to a 10 percent polyacrylamide gel with a 3 percent stacking gel prepared according to the method of Laemmli (16). Electrophoresis was for 9 hours at 150 volts/cm. The gel was then stained in 10µg/ml ethidium bromide and photographed under ultraviolet light. NCDV - Nebraska Calf Diarrhoeal Virus; NI - Northern Ireland bovine rotavirus; Wa - Human cultivable isolate (30); SA-11 - Simian 11 virus. Strain description of wild isolates is by the method of Rodger and Holmes (23). BO, bovine; CN, canine; EQ, equine; HU, human.

Figure 2: Northern Blot sequence analysis of rotavirus genomic RNA's utilising ^{32}P-cDNA mixed probes specific for the genome of SA-11 virus. For details of the hybridisation procedure see refs. 1 and 24. Arrows indicate examples of isolates where genes 4 and 5 show no detectable homology with the SA-11 probe.
Hybridisation was at 52^{o} in the absence of formamide (34% base mismatch tolerated).

Figure 3: Northern Blot sequence analysis of rotavirus genomic RNA's utilising ^{32}P-cDNA mixed probes specific for NCDV. For details

of the hybridisation see refs. 1 and 24. The arrow in (a) indicates evidence for the absence of homology between gene 4 of NCDV and the equivalent gene of the four wild-type bovine strains examined.

(a) Low stringency hybridisation (0% formamide).

(b) High stringency hybridisation (50% formamide).

Figure 4: Sequence analysis using a ^{32}P-cDNA mixed probe specific for the wild-type canine isolate CN/DUN/59/80. For details of hybridisation see refs. 1 and 24. The arrow in (a) indicates the absence of homology between the probe and gene four of the human strain Wa.

(a) Low stringency hybridisation (0% formamide, 52^{o}).

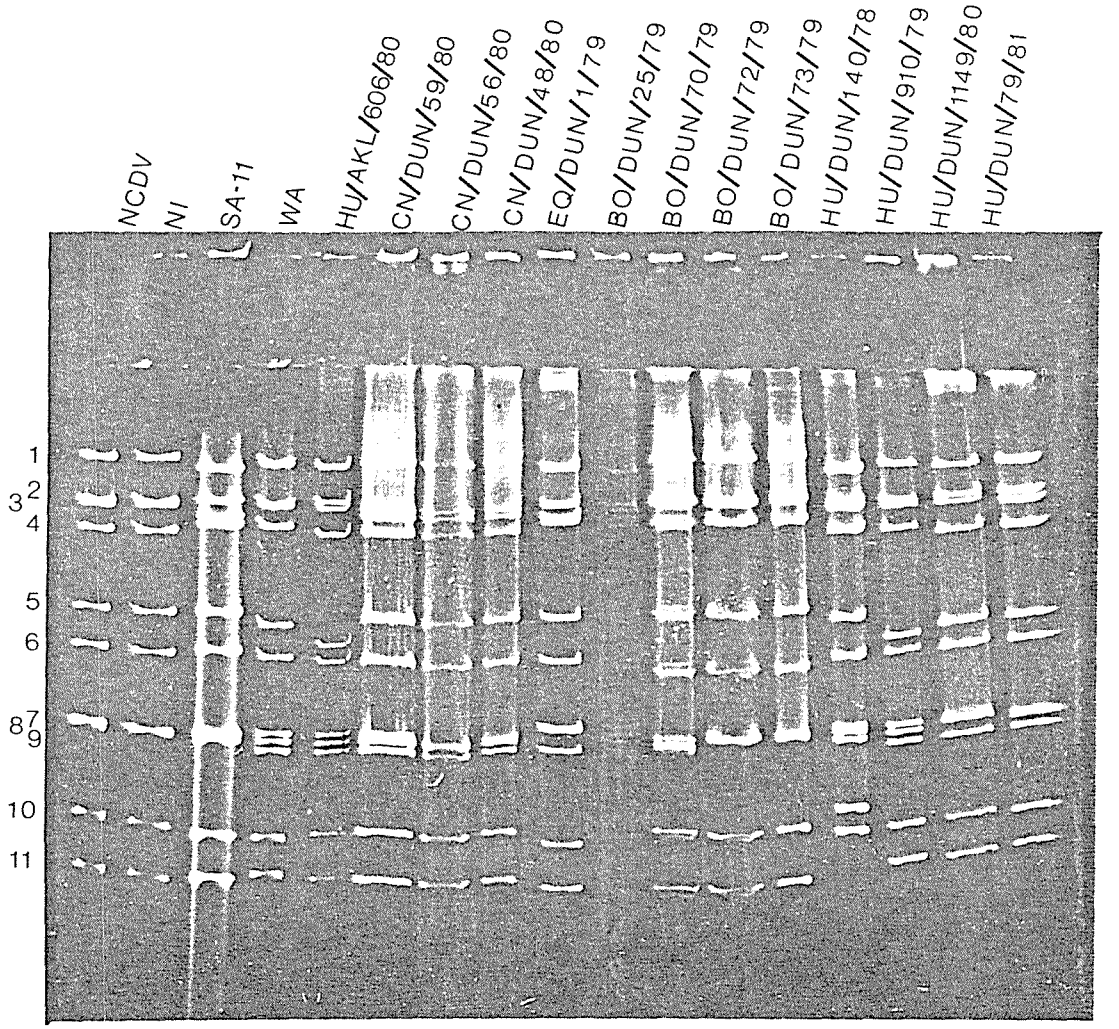
(b) High stringency hybridisation (50% formamide, 52^{o}).

Figure 5: Sequence relationships between human and bovine rotaviruses.

(a) Ethidium bromide stained gel showing electrophoretic separation of the genomes of a series of human rotavirus isolates collected between 1975 and 1980. 'Short' electropherotypes (channels 4 and 4, 8, 9) are those in which the mobility of the smallest segments differ as a result of a major change in the mobility of segment eleven (see ref. 8).

(b) The gel shown in (a) was blotted onto DBM paper and hybridised with a ^{32}P-cDNA mixed probe specific for the cultivable bovine strain NCDV. Low stringency hybridisation (10% formamide, 52^{o}).

(c) High stringency hybridisation (50% formamide, 52^{o}) corresponding to (b) above.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BO/DUN/25/79
BO/DUN/70/79
BO/DUN/72/79
BO/DUN/73/79
HU/DUN/140/78
HU/DUN/910/79
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Fig. 1

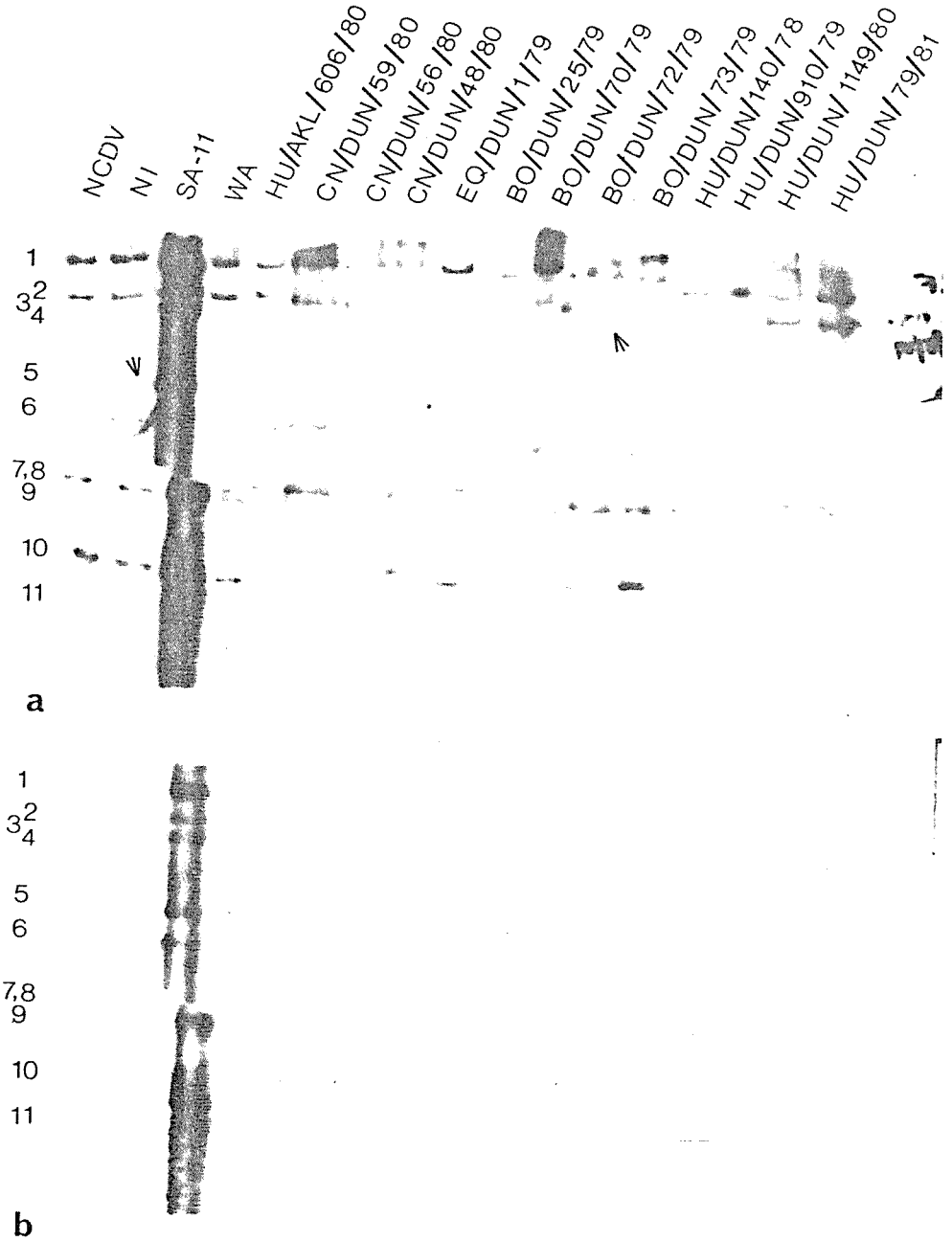
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Fig. 2

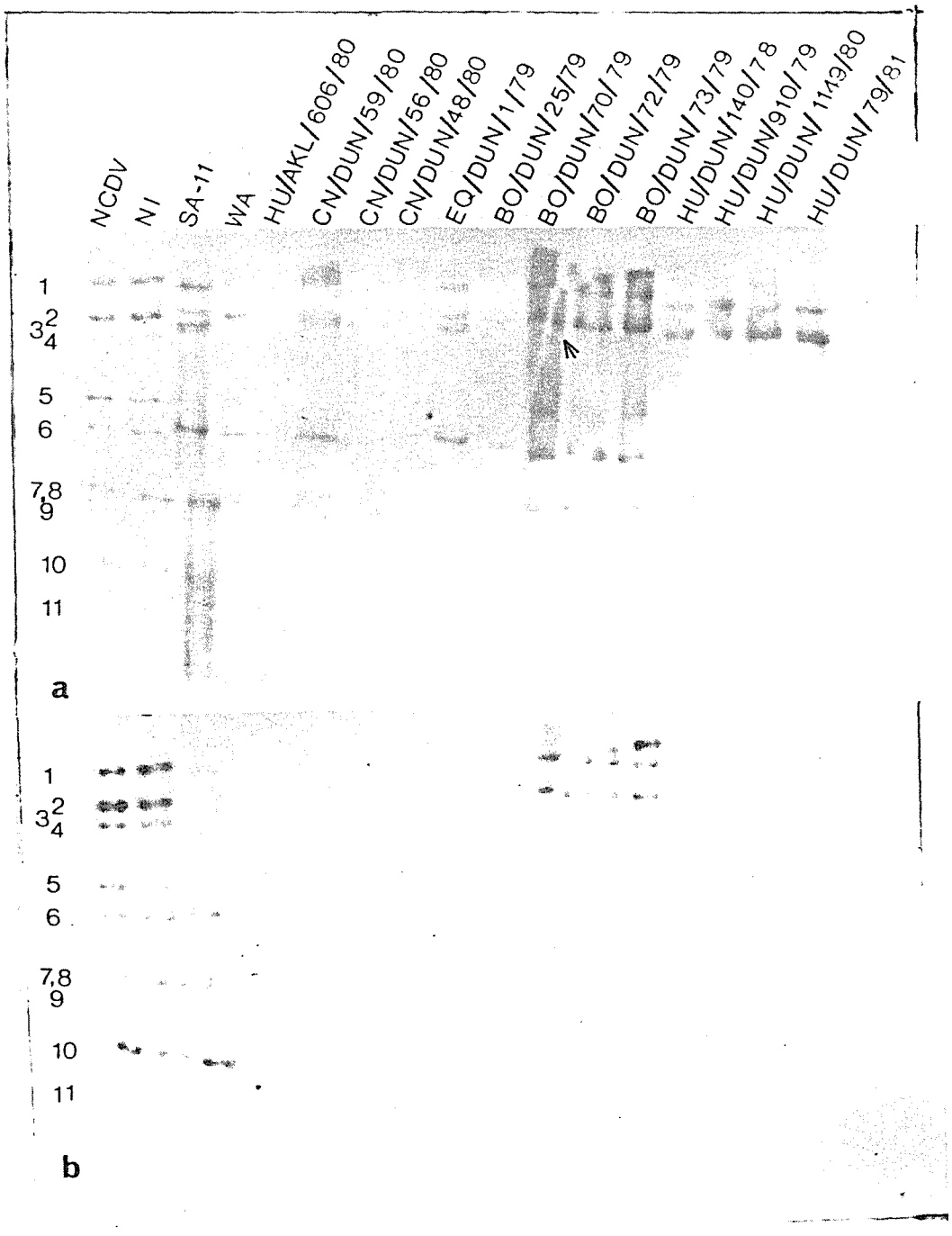
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Fig. 3

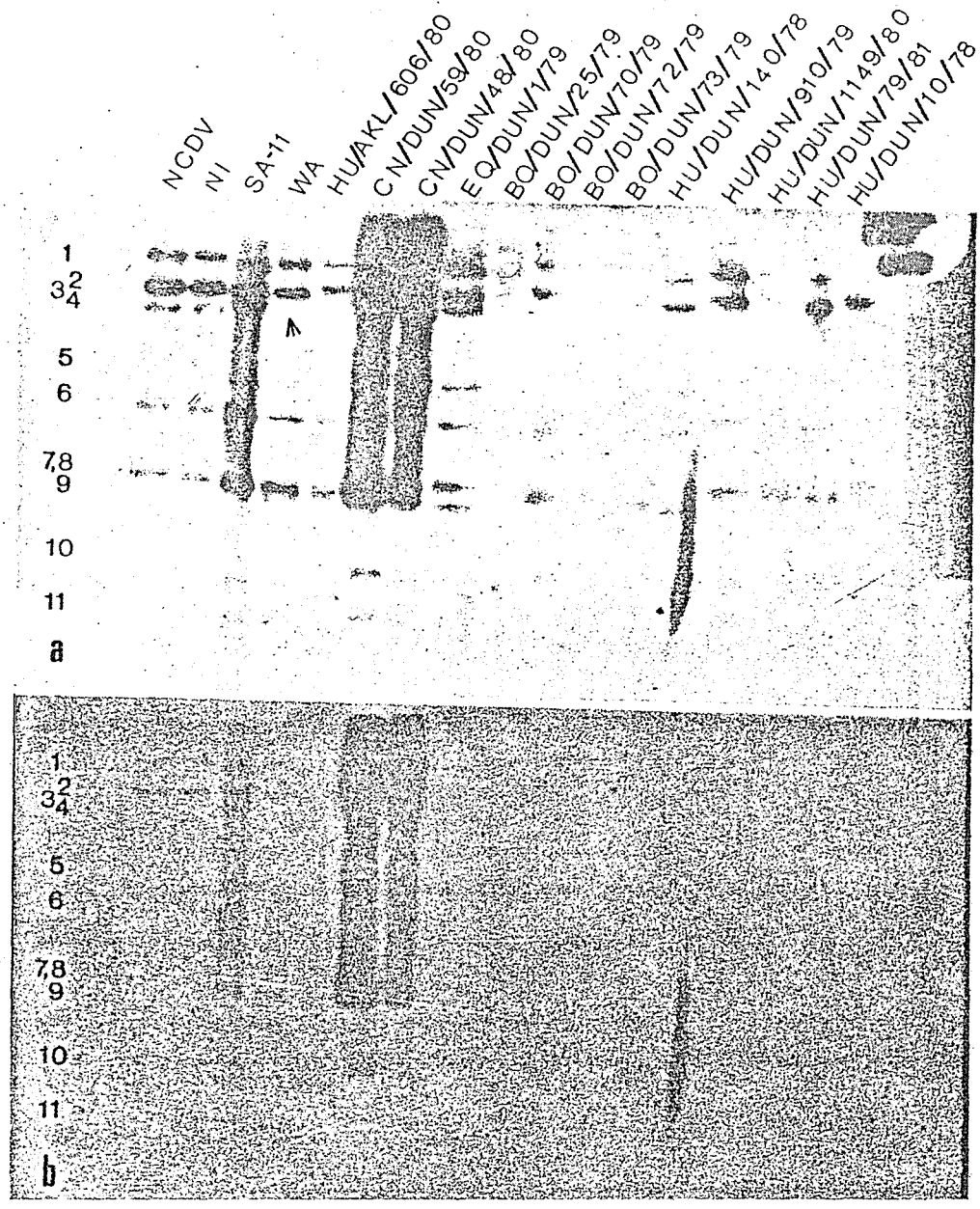
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Fig. 4

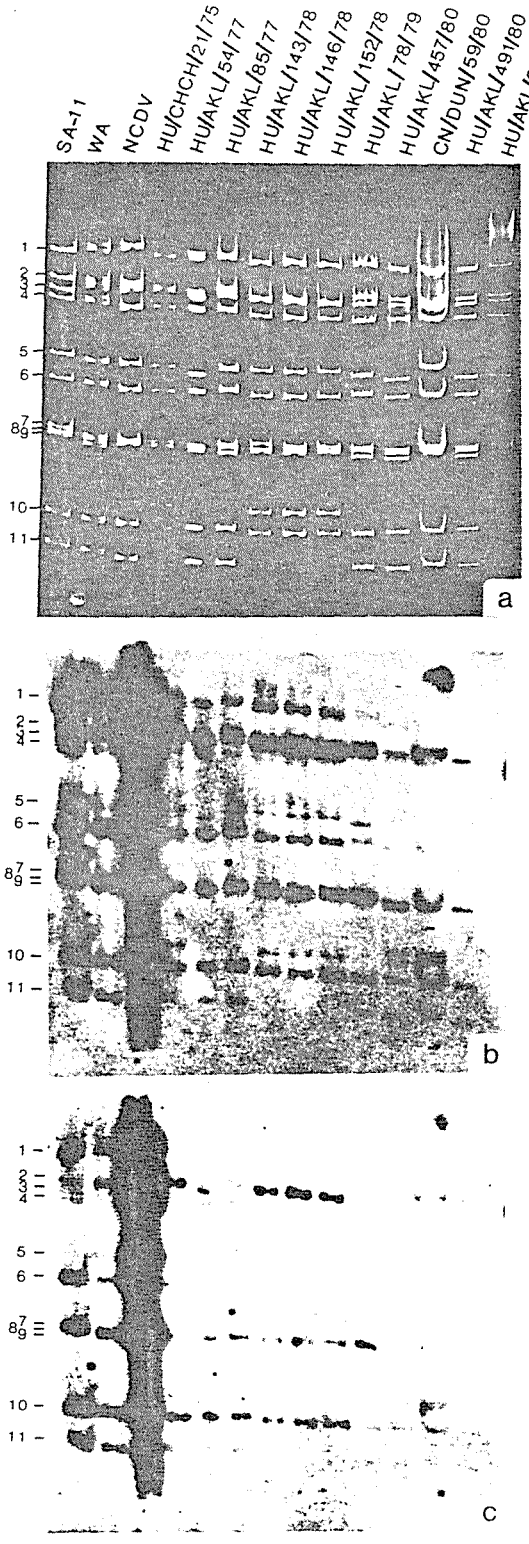
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Fig. 5

THE ISOLATION OF ROTAVIRUSES FROM CALVES, FOALS, DOGS AND CATS IN NEW ZEALAND

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ABSTRACT

The incidence of rotavirus in calves, foals, dogs and cats in the Dunedin urban and rural areas was investigated using electron microscopy and enzyme-linked immunosorbent assays. Of the 283 faecal specimens examined, 26% were positive for rotavirus.

Comparison of the genetic electropherotypes was made by separating the viral dsRNA segments using polyacrylamide gel electrophoresis.

Introduction

Rotaviruses are recognised as the major cause of gastroenteritis of the young in many different animal populations. Rotavirus was first established as a disease causing agent in mice (Adams, et al., 1967) and subsequently in calves (Mebus, et al., 1969), piglets (Rodger, et al., 1975), foals (Flewett, et al., 1975), lambs (McNulty, 1976), deer (Tzipori, et al., 1976), other animals (Holmes, 1979) and humans (Bishop, et al., 1973).

In New Zealand rotaviruses were first reported from humans (Barnes, 1975) then in calves (Burgess, et al., 1976), piglets and foals (Durham, et al., 1979).

Rotaviruses from different hosts have a number of similar features. They are morphologically similar and share a common group antigen (Kapikian, et al., 1974) cause similar clinical symptoms and can induce symptomatic infection in experimentally controlled cross-infection studies (Mebus, et al., 1976; Tzipori, 1976b). These features and the incidence of rotaviruses in human and animal populations has led to the inquiry of whether there may be a zoonotic spread of rotaviruses. Cats and dogs, which have a high contact rate with young children would be obvious sources of rotavirus infection in man.

Rotaviruses of cats and dogs have received very little attention in the overall study of rotaviruses. In 1978 McNulty, et al., reported antibodies to rotavirus in approximately 80% of both dog and cat serums tested, indicating that rotavirus is a common infectious agent of both these animals. Dagenais, et al., (1980a) reported rotavirus antibodies in cats and dogs in Belgium and rotavirus in faeces of dogs suffering from diarrhoea (1980b). Tzipori (1976b) passaged human rotavirus in 2 week old pups yielding large quantities of virus without causing clinical disease.

Using the enzyme-linked immunosorbent assay (ELISA) and electron microscopy (EM) we have studied the incidence of rotavirus in dog, cat, calf

and foal populations in the Dunedin and rural areas. Rotavirus was detected in all four animal populations and was confirmed as rotavirus by polyacrylamide gel electrophoresis (PAGE) of the segmented double stranded (ds) RNA genome. This is the first report of the dsRNA electropherotypes for cat and dog rotavirus isolates and also compares the electropherotypes of calf and foal rotaviruses with some of the local human rotavirus types.

Materials and Methods

Specimens

Faecal specimens were collected from symptomatic and asymptomatic animals over a two year period - 1979-1980. Calf and foal specimens were collected from a number of dairy farms and stud horse breeding properties on the Taieri Plain and from the Invermay Agricultural Research Centre of the Ministry of Agriculture and Fisheries. Cat and dog specimens were collected from cat and dog breeders, the S.P.C.A. and local veterinarians. The human rotavirus specimens were collected as part of an ongoing study of gastroenteritis in childhood in Dunedin.

EM

For EM examination, faecal material was suspended in phosphate buffered saline and dried on formvan coated grids and negatively stained with 1% phosphotungstic acid. Specimens were visualized using a siemens electron microscope.

ELISA

An indirect solid phase system was used. Faecal suspensions in PBS were dried on microtitre wells and with washes between steps, were reacted with rabbit anti-SA serum and secondly with horse-radish peroxidase conjugated sheep anti-rabbit IgG. Controls to check for nonspecific binding of sera, giving false positives, included pre-immune rabbit sera and sheep anti-

rabbit conjugate reacted directly with faecal material. Orthophenylene diamine H_2O_2 substrate resulted in colour reactions of different intensities depending on the amount of conjugated antiserum bound. The resulting colour reactions were read by eye, a positive result being when the test well was of stronger intensity than control wells.

RNA Extraction and PAGE

The method used for viral RNA extraction was essentially that of Croxson and Bellamy (1981). A faecal suspension in Tris-acetate buffer and cold lithium dodecyl sulphate was clarified by low-speed centrifugation and the virus pelleted by ultracentrifugation. The pellet was resuspended in electrophoresis sample buffer and heated to 80°C for 2 minutes prior to electrophoresis.

Rotaviral dsRNA segments were resolved in 10% polyacrylamide slab gels using the laemilli discontinuous system (1970). A 5% stacking gel was used and RNA was applied in 10-20µl volumes per channel (0.5µg to 2µg RNA). Electrophoresis was for 7 hours at 150 V. Gels were stained with ethidium bromide (1µg/ml water) for 30 minutes and photographed in ultraviolet light on a ChromatoVue ultraviolet illuminator screen with Kodak Tri-x-pan film.

Results

Survey

Over the two year period a total of 283 faecal specimens were screened by EM for virus particles and ELISA for rotavirus antigen. 26% were found to be positive with 50% of these showing clinical evidence of disease.

The overall incidence of rotavirus in calves on dairy farms in the Taieri Plains was low (13%). Reports of scouring calves of dairy herds in the surveyed area were common but in the majority of cases symptoms resolved rapidly. Where rotavirus was present on a property there was usually a history of scouring in calves for a number of years previously.

Specimens from foals were collected largely from properties with a previous history of scouring. The scouring developed usually within the first two weeks of life and persisted for 2-5 days. Scouring was particularly a problem for stud breeders with a high percentage (47) proving to be positive for rotavirus.

Specimens from dogs were collected mainly from local veterinarians, SPCA and local breeders. Of the 13 animals positive for rotavirus, 5 had severe scouring. These were greyhound pups all of the same litter. Symptoms developed one week post weaning and persisted for 5 days with fever and convulsions. There were no deaths.

In cats the disease never appeared as a severe illness but as loose stools that lasted for 1 to 2 days. Of the 33 animals that proved to be positive only 10 showed symptoms of disease. Infected kittens ranged in age from 15 to 58 days. One mother cat whose kittens were rotavirus positive was also positive.

PAGE

Rotavirus dsRNA is in eleven segments which may be resolved by PAGE. In some cases 2 or 3 of these segments having similar molecular weights may migrate the same distance upon electrophoresis and hence stain as a single band.

Upon electrophoresis the electropherotypes of the various animal rotavirus isolates resolved quite different patterns. Dog rotavirus segments separated into 10 distinct bands, cat rotavirus into 8 bands, foal rotavirus into 10 bands and calf rotavirus into all 11 bands.

The cat and the dog genomes appeared to be very similar in the first six segments but quite dissimilar in the rest. The dog rotavirus genome migration pattern appeared to be very similar to the human patterns. The two calf rotavirus isolates did show some differences in migration pattern, even though they were isolated at the same time in close geographical prox-

imity. The calf and foal isolates were distinctly different in their migration patterns.

Discussion

Electropherograms of rotavirus types of the four animal species that were here examined all show some dissimilarity in terms of migration distance of various dsRNA segments. They are also dissimilar to the human electropherotypes. To date we have resolved 11 different rotaviral dsRNA electropherotypes - 5 human, 3 calf, 1 dog, 1 cat and 1 foal. The significance of interspecies and intraspecies genome variability has not yet been fully determined. It is possible that intraspecies differences only indicate differences in the degree of virulence or nonstructural proteins of the virus and that interspecies differences reflect host range limits. The *in vitro* reassortment of genome segments between types shown by Matsuno, *et al.*, (1980) indicates that reassortment of genetic segments between "wild" rotavirus types is theoretically possible. Such reassortment may allow the emergence of a new type with a new host range or a change in virulence within the existing host range. Rotavirus types may then be quite variable with changes of types prevalent in a community over a period of time being quite common. Although our results show no direct evidence of reassortment of genetic segments between types the number of different types being resolved by PAGE, the capability for *in vitro* segment reassortment, the ability of human rotavirus to cause clinical infection in young animals and the incidence of rotavirus in young animals in close contact with young children all give strong support to rotavirus being able to cause zoonotic disease.

A common trend noted with calf and foal scours caused by rotavirus was a history of scours on the same property over a number of years. Two possible explanations may account for this. The virus is highly stable and may

exist from calf and foal seasons in particular holding paddocks or rearing pens. Adult animals may also be carriers of the virus spreading infection to young animals each new season.

A continued surveillance on the electropherotypes existence in a community needs to be made in order that any changes of type may be correlated with any changes in disease patterns. Hybridization techniques and cloning of particular segments of rotaviral RNA will allow for a clear understanding of the degree of relationship of different types being observed and of the importance of differences of electrophoresis migration of particular segments.

Acknowledgements

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Rotavirus infection in New Zealand

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Summary

Rotavirus infection is commonly found in young infants admitted to hospital with gastroenteritis. An enzyme-linked immunosorbent assay (ELISA) for virus diagnosis is described and the results of testing stool specimens from 497 children with gastroenteritis, 192 neonates and 247 asymptomatic six month old infants are presented.

Rotavirus infection was found in 45 percent of all children with gastroenteritis but only in 4.7 percent of neonates and 2 percent of asymptomatic infants.

These results do not support the proposal that children in our community have a high incidence of subclinical infections.

NZ Med J 1982; 95: 67-9

Introduction

Acute gastroenteritis is common in children and often leads to hospital admission. Prior to 1973 the aetiology of most cases of acute vomiting and diarrhoea in young children was unknown but now rotavirus has been established as a major agent.[1,2] Rotavirus belongs to the Reoviridae and is related to viruses causing epidemic diarrhoea of infant mice, the Nebraska calf virus, a Simian rotavirus (SA_{11}), the O virus of sheep and similar viruses in piglets, foals and birds.[3]

Rotavirus gastroenteritis has been reported in New Zealand children previously.[4-7] Other authors have reported antibodies to rotavirus in older children and adults[8] and the existence of more than one serotype.[9] The present studies were carried out to obtain further information on the distribution of infection in New Zealand in the period 1977 to 1980.

Materials and methods

Virus detection by ELISA. An enzyme-linked immunosorbent assay (ELISA) has been developed for rotavirus diagnosis in faeces samples. A double sandwich method, similar to that described by Middleton et al[10] for rotavirus diagnosis by radioimmunoassay has been adapted to ELISA. For this purpose guinea pig and rabbit antisera were prepared by immunising experimental animals with purified human rotavirus previously identified by electron microscopy (EM) during a gastroenteritis outbreak in late 1976. The virus was purified by differential centrifugation and banding in a caesium chloride gradient.[10] Sheep antirabbit serum was conjugated with horseradish peroxidase by the technique of Nakane and Kawoi.[11] Microtitre plates (S-MRC-96) were used in place of polystyrene tubes described by Middleton et al.[10] The substrate used was orthophenylene diamine and any colour change was either read by eye or by a spectrophotometer at 492 nm. All tests were carried out in duplicate with rotavirus negative rabbit serum as a control.

Clinical material. Specimens of faeces were obtained from infants and young children admitted to Wakari and Dunedin Hospitals with gastroenteritis, between June 1977 and September 1980. Other fecal specimens were provided from cases in the community and from young patients of colleagues throughout New Zealand.

Faeces specimens were also collected from neonates in the Queen Mary Maternity Hospital between the 5th and 7th day of life both at a time of a rotavirus epidemic in the community and in inter-epidemic periods between 1978 and 1980.

The frequency of subclinical infection in the community was studied by examining single faeces specimens collected from 6 month old infants, living at home, over a 12 month period between 26 March 1979 and 25 March 1980. Infants with rotavirus in their faeces were followed up to ascertain if they had any clinical symptoms, and further specimens were taken to determine the duration of excretion.

Results

Comparison of ELISA with EM. The ELISA technique was compared for the sensitivity with EM using known concentrations of purified virus and was found to be at least 18 times more sensitive. Previous experiments have shown that ELISA and the radioimmunoassay technique (RIA) are equally sensitive. (Holdaway, Kalmakoff, Todd, in unpublished) In a trial with 16 positive faeces specimens, diagnosed by EM, and 50 negative specimens, there were no false positive or false negative reactions by ELISA.

Rotavirus in childhood gastroenteritis. From June 1977 until September 1980 faeces specimens were collected from 497 children with gastroenteritis referred for rotavirus diagnosis. A majority, 252, were admitted to the paediatric unit at Wakari Hospital (233 children) or were treated at home in Dunedin. A further 245 specimens were referred from children living in other centres throughout New Zealand. All specimens were tested even though some appeared to be normal stools. In the Dunedin series, 112 or 44 percent of the specimens were positive for rotavirus and 46 percent of the stools referred from other centres. No attempt was made to examine the specimens of faeces for other non-culturable viruses. Possible bacterial pathogens were isolated from 21 of the

233 children admitted to Wakari Hospital and included 5 serotypable E. coli, 11 Salmonella species, 4 Shigella species and 1 Campylobacter.

Seasonal incidence. Nonbacterial gastroenteritis is predominantly a winter disease in New Zealand. Figure 1 shows the distribution of cases by month over the 40 month period.

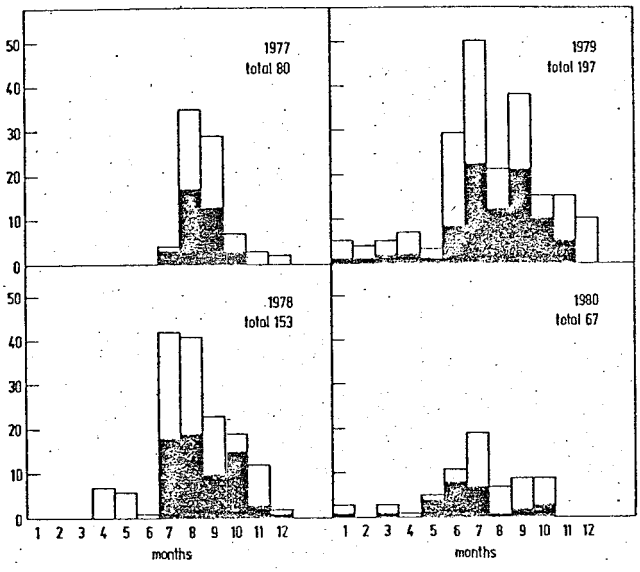

Figure 1.—Results of analysis of faeces specimens received over a 40 month period. ELISA positive specimens indicated by shaded areas.

A similar seasonal incidence was shown when only Dunedin cases were considered. The months showing the highest frequency of cases of gastroenteritis were also those in which most rotavirus infections were identified. In some months 75 percent of faeces specimens were positive for rotavirus.

Age distribution. The majority of children admitted to hospital with gastroenteritis were less than 2 years of age, 217 of the 233 in Dunedin, with the greater number less than 1 year. Potential bacterial pathogens were isolated from children of all ages but rotavirus was uncommon over the age of 4 years. Only three of the 123 rotavirus positive specimens in Dunedin came from children more than 4 years of age.

Respiratory symptoms. Respiratory symptoms were frequent in children with gastroenteritis. In a study of 82 children with vomiting and diarrhoea, 43 percent of the children with rotavirus in their faeces had respiratory symptoms and 45 percent of the 22 children with rotavirus negative faeces. The majority of the affected children had otitis media.

Neonatal infections. In 1978 faeces samples were obtained from 141 neonates aged 5 to 7 days in the Queen Mary Maternity Hospital. No rotavirus was found in 80 specimens collected between May and early June when there were few cases of gastroenteritis in the community. Only one positive identification was made in an asymptomatic infant from the 61 specimens collected during the height of an epidemic in August and September of the same year.

In 1979 faeces specimens were sent from 21 neonates with diarrhoea but only 1 was positive. Further specimens were collected in 1980 from 30 infants aged 5 to 7 days being nursed in the special care baby unit. Although none of these infants had diarrhoea, 7 were positive for rotavirus including 6 of 18 specimens collected in July when the infection was prevalent in the community.

Of the 192 neonatal specimens examined, only 9(4.7 percent) were positive but 7 of these were collected in the special care unit in 1980. It is worth noting that only 1 of the 9 infected infants was receiving breast milk.

Six month infant study. Faeces were collected from 247 six-month old infants over the 12 month period. Only 5(2 percent) of the specimens were positive for rotavirus. Three of the infants were asymptomatic, one developed diarrhoea shortly after discharge from hospital and was clearly a nosocomial infection, and the fifth infant remained asymptomatic for 12 days, during which time he continued to excrete virus, and then developed diarrhoea. His faeces still contained rotavirus at the time of the onset of symptoms and no other virus or pathogenic bacteria was identified.

Discussion

Rotavirus infection was first identified in Dunedin children by electron microscopy.[4,5] The ELISA test is now used exclusively for virus diagnosis since it is less time consuming and more suited to screening large numbers of specimens. Specimens have been exchanged with the Melbourne group and our methods of detection compare favourably. (Bishop, Barnes personal communications).

The results presented in this paper are similar to those reported by other workers,[2, 7, 12] who found that hospital admissions occur predominantly in younger children. The reasons for the winter seasonal nature of the infection is not fully understood.

It has been proposed that there is a high incidence of subclinical infection. Studies of neonatal nurseries in London,[13] and Sydney[14] showed that 32.5 percent and 49 percent respectively were excreting virus asymptomatically. In our study only 1.2 percent of 162 neonates were excreting rotavirus in the first two years but in 1980 this was increased to 27 percent. The presence of diarrhoea in the neonates studied in 1979 was not associated with an increased frequency of rotavirus excretion.

In one of the hospitals studied by Murphy and his colleagues,[14] no cases of rotavirus infection occurred amongst 40 neonates, whereas in other hospitals nearly half of the babies were infected. This, along with our study, suggests that rotavirus infection is related to differences in methods of child care, rather than a specific feature of the virus. A possible factor causing a decrease in infection in our neonates may be the high frequency of breast feeding (75 percent). Chrystie and his colleagues[13] noted infection occurred in 22 percent of breast fed and 58 percent of formula fed infants. The majority of infections (89 percent) occurred in formula fed babies in our study.

In the special care baby unit at Queen Mary Hospital, 7 of the 30 specimens collected in mid 1980 were positive for rotavirus. These infants were premature or sick, requiring handling by several adults. The majority of infections, 6 of 7, occurred at a time when rotavirus was common in the community suggesting that it was introduced into the nursery by adults.

In the six-month old infant study we obtained about 20 specimens a month from a population representing approximately 15 percent of total births in Dunedin. Rotavirus was found in 2 percent of the infants but identifications only occurred at times when clinical cases were being admitted to hospital. This does not support the premise that subclinical infection is common in infants throughout the year.

Infection may not be maintained in the community by children alone. Several studies have indicated that outbreaks of symptomatic rotavirus infection have occurred in adults including parents of young children,[15] patients in a geriatric hospital,[16] and others attending an outpatient clinic.[17] Infection in adults may be almost as frequent as in children.

Gastroenteritis is often referred to as "gastric flu" by the lay public, indicating a recognition of the assocation

between vomiting, diarrhoea and respiratory symptoms. In our studies more than 40 percent of patients had respiratory symptoms within a week of the onset of gastroenteritis. It is suggested that rotavirus may involve the respiratory tract but like other workers[18] we have been unable to identify the virus in respiratory secretions.

The Dunedin study results show that of approximately 840 admissions to the paediatric unit at Wakari Hospital each year, 1 in 6 are admitted because of gastroenteritis and 44 percent are associated with rotavirus infection. Extrapolating these figures to the whole of New Zealand, there may be more than 2000 children requiring hospitalisation annually and 2 or 3 may die.[6] Rotavirus is a major cause of ill health in New Zealand and further studies are needed to define the sources and mode of spread of infection.

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Reprints. Requests for reprints to Associate Professor M. D. Holdaway, Department of Paediatrics and Child Health, University of Otago Medical School, PO Box 913, Dunedin.

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Rotavirus infection in Otago: a serological study

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Summary

A method for measuring rotavirus antibody in human sera has been established using enzyme-linked immunosorbent assay (ELISA). A Simian strain of rotavirus (SA11) was used as the antigen. Serum eluted from dried blood spots on good quality chromatography paper was found suitable for analysis.

Paired serum samples from children with gastroenteritis have shown a brisk antibody response in association with the presence of rotavirus in the faeces. Community studies indicate that although all older children and adults tested have detectable antibodies to rotavirus, there is a significant rise in the number of individuals with high titre antibody in the child bearing age group, after which the levels diminish. This finding suggests that repeated infections occur throughout childhood and early adult life.

NZ Med J 1982; 95: 110-2

Introduction

Rotavirus has been established as the major aetiological agent for acute gastroenteritis of infants and children.[1, 2]

In a preceding paper the seasonal occurrence and age distribution of rotavirus in New Zealand was studied and showed that hospital admission for rotavirus infection occurred mostly in children less than 2 years of age during the winter months.[3] It has been suggested that subclinical infection in children maintains the virus in the community between epidemics. However, adults can be severely affected and there are reports of outbreaks in a geriatric hospital,[4] in parents of infected children,[5] and in adults attending an outpatient clinic.[6] Widely differing titres of rotavirus antibody have been detected in all age groups, but more specifically in young children.[7, 8] This suggests that either humoral antibody offers no protection to recurrent infection or that there are several different strains of rotavirus.

To determine the immunological status of the Otago population, a serological study has been carried out using the enzyme-linked immunosorbent assay (ELISA). The results of assays of humoral antibody to rotavirus in a wide cross-section of the community are presented.

Methods

Rotavirus serology by ELISA. A Simian strain of rotavirus (SA11, the virus was obtained from Dr R. Schnagel, Microbiology Department, University of Melbourne, (now at La Trobe University, Melbourne), propagated in primary monkey kidneys cells (Commonwealth Serum Laboratories) was used as antigen. Sonicated virus infected tissue culture cells were diluted 1/240 in phosphate buffered saline (PBS) and dried onto microtitre wells (Limbro S-MRC-96 polyvinyl trays) at 37° overnight. Uninfected cell cultures were used as a negative control antigen. The plates were blocked with 0.1 percent bovine serum albumin in phosphate buffered saline (BSA/PBS) and all subsequent washings were carried out with this solution. The serum dilutions were incubated for 2 hours at room temperature, the plates washed and horse radish peroxidase conjugated sheep antihuman globulin, prepared in this laboratory by the technique of Nakane and Kawoi,[9] was added. The plates were further incubated for 2 hours at 46°C. The substrate orthophenylene diamine, was then added and incubated for 30 minutes at room temperature, in the dark. All sera were tested in duplicate and the results were read by eye or spectrophotometrically at 492 nm.

Serum eluted from dried blood spots on chromatographic paper (Schleicher-Schull Numbers 903 or 2992) was found satisfactory for analysis. The serum was eluted from 2 spots, each measuring 14 mm in diameter with 1.5 ml of BSA/PBS. There was a small drop in titre (2 to 4 fold) when the blood spot serology was compared with a liquid sample but this was not found to be significant in practice as titres were generally high in response to infection. Blood spot samples are now routinely used in our laboratory.

Serology specimens

Paediatric inpatients. Paired blood samples were obtained from children presenting with acute gastroenteritis to the paediatric unit at Wakari Hospital. The acute phase specimens were obtained as soon as possible after the child's admission to hospital. Convalescent specimens were collected 10 to 30 days later.

Antenatal serology and community studies. In order to study the nature of rotavirus infection elsewhere in our community, 46 sera were obtained from women attending the Antenatal Clinic at Queen Mary Hospital. We were also able to obtain samples from family groups in the Port Chalmers community collected between late 1973 and 1974.[10] The individuals in the study ranged in age from 5 to over 70 years. A further group of sera were obtained by random selection of 222 blood samples from the total collection of 2000 obtained during the study of the adolescent and adult population of the south Otago town of Milton in 1978.[11] The specimens tested came from individuals aged between 20 and 70 years.

Data analysis. The results of the Port Chalmers and Milton studies were analysed by χ^2 to determine which age group deviated most from expected antibody levels, testing the null hypothesis that there was no relationship between age and antibody levels. A high titre was arbitrarily defined as equal to or greater than 1/2048.

Results

Serology in paediatric inpatients. Paired sera from 18 children admitted to hospital with acute gastroenteritis were available for analysis. Eight patients with faeces positive for rotavirus had a greater than four fold rise in antibody titre by ELISA. Acute phase specimens had an initial titre of less than 1/16 in 7 of these children and 1/64 in 1. Convalescent sera had titres of from 1/64 to 1/2048. In all cases there was a greater than four fold rise in titre from acute to convalescent specimens.

In the five patients whose faecal specimens were negative for rotavirus by ELISA, one demonstrated a rise in antibody of 1/16 to 1/64, another a fall in titre from 1/512 to 1/8 and the remainder had titres of less than 1/16 in the acute and convalescent specimens.

The remaining five pairs of sera were obtained from children with gastroenteritis but without accompanying faeces specimens. Three of these pairs had a greater than four fold rise in titre, one had no change from an initial titre of less than 1/16 and one had a four fold drop in titre.

Antenatal serology. Serology of blood samples taken from 46 women in the first trimester showed that all had antibodies, some to a very high titre (Figure 1).

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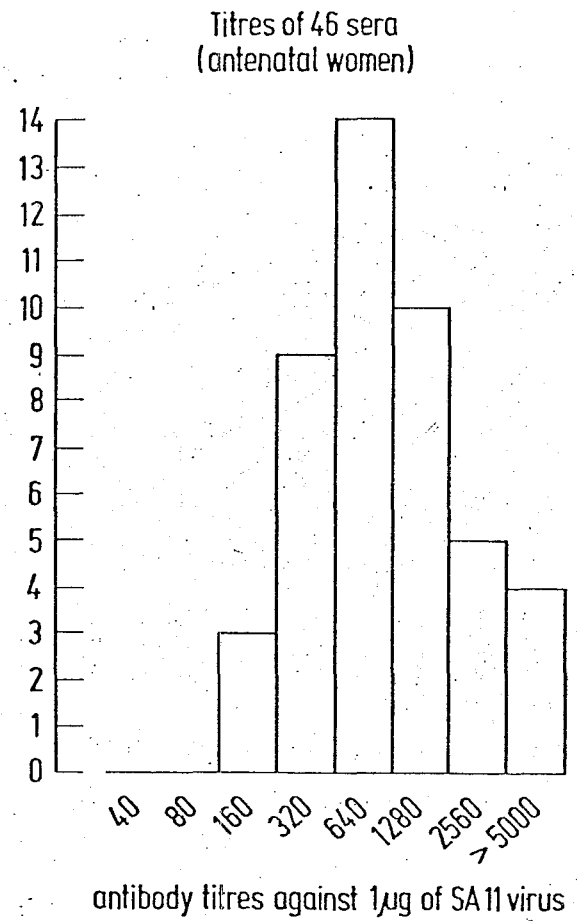

Figure 1:—Rotavirus antibody in 46 pregnant women in the first trimester.

Port Chalmers and Milton serology. The results of the serology tests in the two Otago communities of Port Chalmers and Milton are illustrated in Fugre 2.

Figure 2A illustrates the results of blood samples from 148 residents of the harbour town of Port Chalmers obtained between the end of 1973 and early 1974. All had detectable antibody but there was a steady increase in the proportion of individuals with a high titre (arbitrarily defined as ≥ 1/2048) up till the age of 30, after which was a decline, with a further small rise in the 60 and under 70 year old age group. Applying the χ^2 test, there was a clear excess of high titres in the young adult group.

In Figure 2B, a similar pattern for young adults is shown in the study of residents of the south Otago town of Milton in 1978. No children were included in this study.

Discussion

Our ELISA technique for measuring serological response uses the Simian strain of rotavirus SA11 which shares a common antigen with human rotavirus but unlike the latter can be grown in tissue culture. The sensitivity of this test has allowed the development of a technique using serum eluted from blood spots on good quality chromatography paper.

Most children admitted to hospital with rotavirus infections had negligible antibodies in the acute phase but showed a rapid rise in titre in the convalescent specimen. Children with faeces negative for rotavirus usually showed no serological response although one demonstrated a relatively slight rise of 1/16 to 1/64 and another a falling titre. It was not possible to be certain whether rotavirus was a prime cause of diarrhoea in these two children.

The virus specific antibodies in the sera from the pregnant women study confirms the work of others[7, 8] who found that adults usually have a high titre of humoral antibody. The occurrence of rotavirus infection in neonates[3] suggests that passive immunity is not acquired via the placenta. Humoral antibody may only be of secondary significance in protecting individuals since both man and animals may be reinfected in the face of high titre antibodies in the serum,[6, 12] suggesting the importance of local immunity in this infection.

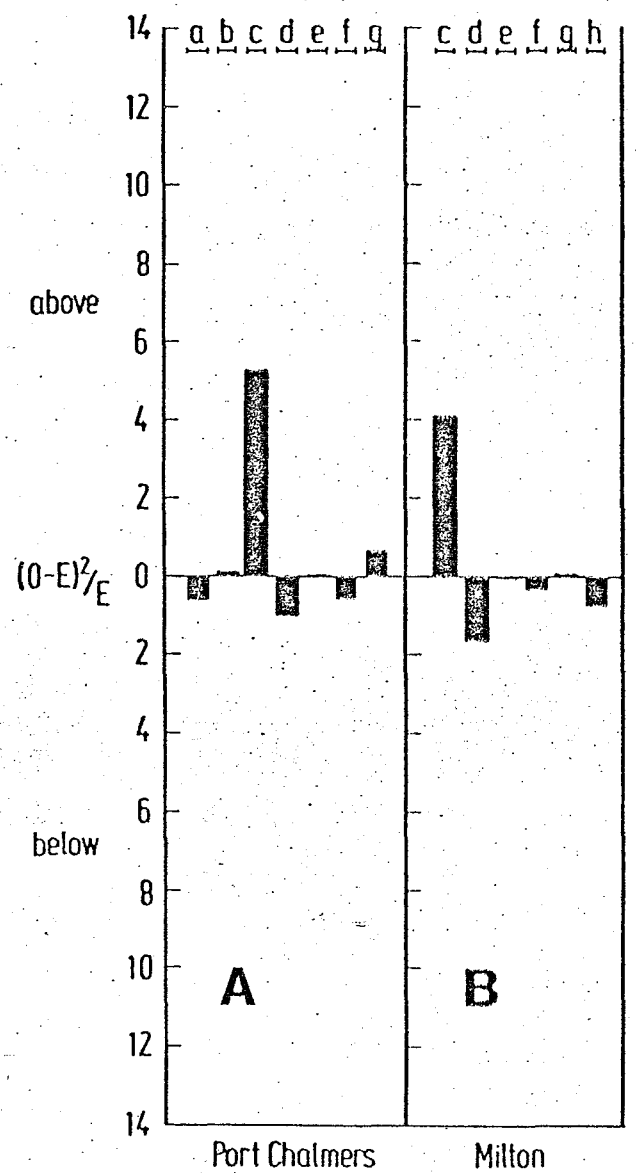

Figure 2.—Comparison of frequency of high titre antibody to rotavirus in different age groups in (A) Port Chalmers, (B) Milton.

Age groups a = 0 <10 b = 10 <20 c = 20 <30
d = 30 <40 e = 40 <50 f = 50 <60
g = 60 <70 h = >70 years.

Our community studies have revealed a high level of humoral antibody against rotavirus in all ages but especially in the 20-30 year old age group. The Port Chalmers study shows an increasing level of humoral antibody throughout childhood with a sharp rise in the age group containing the majority of young parents. A similar rise is seen in this age group in Milton, possibly reflecting exposure to their infected infants. Elias[7] and others[8] in serological studies of patients in hospital have suggested that there is a decreasing level of antibodies in the population with increasing age. Hospital based studies introduce a considerable bias in considering patterns of infection in the community. Their

findings may reflect a high nosocomial infection rate, a serious problem with this virus in a children's ward.

The observations in this paper do not indicate whether it is the child or the parent who is primarily infected. A modification of the ELISA technique to measure specific IgM may allow the identification of reinfections in asymptomatic patients who could act as the reservoir of infection in the community. It will also be necessary to define the relative importance of local as against humoral antibody in providing protection from infection.

Finally, it is not clear from the present study whether children and adults are suffering from recurrent infections with the same virus or from different strains to account for the progressive increase in humoral antibody levels in early adult life. The ELISA system described here detects antibody to rotavirus core antigen, common to all strains of rotavirus from man and other animals. It may be necessary to use specific monoclonal antibodies to clearly define the number of rotavirus serotypes causing disease in New Zealand communities.

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These studies were carried out under a grant from the National Children's Health Research Foundation. Brian Schroeder is in receipt of a University Grants Committee postgraduate scholarship. We are grateful to our colleagues in the department of paediatrics and the department of microbiology for their co-operation and help. We would also like to thank Miss P. Johnston for secretarial help.

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